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(54) ANTISENSE NUCLEIC ACID COMPOUND

(57) The present invention relates to an antisense nucleic acid compound which has a nucleotide sequence complementary to at least 8 contiguous nucleotides in the nucleotide sequence of a gene coding for a vascular endothelial growth factor and which inhibits the expression of the vascular endothelial growth factor, as well as to a therapeutic or diagnostic agent for cancers, rheumatoid arthritis, diabetes etc., comprising said antisense nucleic acid as active ingredient. Further, the present invention relates to a method of preventing the expression of the vascular endothelial growth factor, comprising use of an antisense nucleic acid compound which has a nucleotide sequence complementary to at least 8 contiguous nucleotides in the nucleotide sequence of a gene coding for a vascular endothelial growth factor and which inhibits the expression of the vascular endothelial growth factor.

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Description

Fields in Industry

The present invention relates to an antisense nucleic acid compound which has a nucleotide sequence complementary to a nucleotide contained in the nucleotide sequence of a gene coding for a vascular endothelial growth factor and which inhibits the expression of the vascular endothelial growth factor (VEGF), as well as to uses thereof.

Background of the Invention

Extensive research has conventionally been made of tumors and tumor cells as a basis of development in an anticancer drug. As a result, it was found that solid tumors need oxygen and nutrients supplied through blood vessels for their homeostasis in vivo, and without such blood vessels, they cannot be grown to 2 mm or more in diameter in vivo [Basic Science of Cancer, authored by I. F. Tannock and R. P. Hill and translated by Naoyuki Taniguchi, Medical Science International (1993); and "Hatsugan to Gan Saibo" (Carcinogenesis and Cancer Cells)], Cancer Bioscience 3, edited by Toshio Kuroki, Tokyo University Press (1991)).

For this arrival of blood vessels at solid tumors, it was proposed that solid tumor cells produce and secrete a certain factor (a tumor angiogenic factor) to induce blood vessels (J. Folkman, Annals of Surgery, Vol. 175, pp. 409 - 416 (1972)).

Recently, attention has been paid to a vascular endothelial growth factor as one of substances which functions as a tumor-angiogenic factor (N. Ferrara et al., Endocrine Reviews, Vol. 3, No. 1, pp. 18 - 31 (1992)). The vascular endothelial growth factor is the same substance as so-called "vascular permeability factor", and in some cases it is also called "vascular endothelial growth factor/vascular permeability factor". As such factor, 4 kinds of molecular species, which occur depending on the difference of splicing, are found in human.

Recently, it has been found that this vascular endothelial growth factor does not exert direct action (e.g. growth promotion) on solid tumor cells in experiments with cells (in vitro). However, it has been found that this factor promotes the growth of solid tumors in experiments on with animals (in vivo). It has been further revealed that the growth of solid tumors is inhibited by administration of an anti-VEGF antibody to animals. These findings indicate that the vascular endothelial growth factor is a tumor angiogenic factor (K. J. Kim et al., Nature, Vol. 362, April 29 issue, pp. 841-844 (1993); S. Kondo et al., Biochemical and Biophysical Research Communications, Vol. 194, No. 3, pp. 1234 - 1241 (1933)).

From the foregoing, inhibition of the vascular endothelial growth factor leads to inhibition of growth of solid tumor cells, and this should be applicable in the development of anticancer agents. In fact there is a report on a method to use an anti-VEGF antibody. In this prior method, function of the vascular endothelial growth factor (i.e. function of facilitating the growth of solid tumors) biosynthesized via translation of mRNA is inhibited by the anti-VEGF antibody.

However, this prior method is based on the assumption that the vascular endothelial growth factor is present, so it is required for said factor which is not necessary to depress growth of tumor to be produced. Hence, this method cannot be effective until such substance is produced. Further, because the vascular endothelial growth factor itself is biosynthesized without special inhibition, this method is problematic if the specificity and binding ability of the anti-VEGF antibody is poor and the inhibitory action of the antibody is incomplete.

The object of the present invention is to provide a nucleic acid compound (i.e. antisense nucleic acid compound) which completely or almost completely inhibits expression of the vascular endothelial growth factor itself by inhibiting production of the vascular endothelial growth factors at the translation of mRNA, in place of inhibiting the action of the produced vascular endothelial growth factor by use of said anti-VEGF antibody.

Summary of the Invention

To solve the problem, complementary nucleotides (i.e. antisense nucleic acid compounds) towards a gene coding for the vascular endothelial growth factor were screened by using a transcription and translation system derived from a rabbit reticulocyte lysate. Among these, the present inventors found some complementary nucleotides effectively inhibiting the production of the vascular endothelial growth factor, and further confirmed their pharmacological effect on both cultured cells and experimental animals to complete the present invention.

Specifically, the present invention is antisense nucleic acid compounds having nucleotide sequences complementary to at least 8 contiguous nucleotides in the nucleotide sequence of the gene coding for the vascular endothelial growth factor, said antisense nucleic acid compound inhibiting the expression of the vascular endothelial growth factor.

The phrase "gene coding for the vascular endothelial growth factor" herein used means a structural gene defining the amino acid sequence of the vascular endothelial growth factor (including its signal peptide regi n), intervening sequences (introns) located in the structural gene, and upstream nucleotide sequences (promoter, operator etc.) and downstream nucleotide sequences (poly A etc.) involved in the expression of said gene. An example of this gene is

shown in SEQ ID NO: 1.

Said antisense nucleic acid compounds include those compounds which inhibit the expression of the vascular endothelial growth factor to a level of 30 % or less and more effectively to a level of 10 % or less as compared to the expression in the absence of the compound. As those antisense nucleic acid compounds which inhibit the expression to a level of 10 % or less, mention is made of an antisense nucleic acid compounds having a complementary sequence to at least 8 to 10 nucleotides in the nucleotide sequences of SEQ ID NOS:2 to 9. The effect of these antisense nucleic acid compounds can be confirmed in both cultured cell and experimental animal assay systems.

In addition, the present invention is a therapeutic agent comprising said antisense nucleic acid compound as active ingredient.

Further, the present invention is a diagnostic agent comprising said antisense nucleic acid compound as active ingredient.

Furthermore, the present invention is a method of inhibiting the expression of the vascular endothelial growth factor to a level of 30 % or less, which comprises use of an antisense nucleic acid compound having a nucleotide sequence complementary to at least 8 contiguous nucleotides in the nucleotide sequence of the gene coding for the vascular endothelial growth factor.

The phrase "inhibit the expression of the vascular endothelial growth factor" herein used means that the vascular endothelial growth factor is not produced by interfering steps such as translation of mRNA coding step for said factor to produce the vascular endothelial growth factor.

Detailed Description of the Invention

The antisense nucleic acid compound of the invention is obtained in the following manner by designing nucleic acid compounds on the basis of the concept of antisense nucleic acid and then evaluating their effect in terms of the amount of the vascular endothelial growth factor as the protein produced in their presence.

First, the gene which codes for the vascular endothelial growth factor (referred to hereinafter as "VEGF") is sequenced. Thereafter, a nucleic acid compound complementary to a partial nucleotide sequence in said gene is prepared by chemical synthesis etc. This compound is then evaluated by a screening test in a cell-free system whether it can effectively inhibit the production of VEGF or not. A nucleic acid compound having the nucleotide sequence found to inhibit the production of VEGF is the antisense nucleic acid compound of the invention.

In the present invention, if the above synthesized nucleic acid compound inhibits the expression of VEGF to a greater extent than a nucleic acid compound having a random nucleotide sequence, then said synthesized compound can be considered to effectively inhibit the expression. The "random nucleotide sequence" refers to a nucleotide sequence having not more than a statistically expected degree of complementation, and the "antisense nucleic acid compound" refers to a compound capable of inhibiting the expression of VEGF to 30 % or less of that in the absence of the antisense nucleic acid compound.

The effect of some of such antisense nucleic acid compounds is evaluated by using cultured cells. For this, the antisense nucleic acid compounds which inhibit the expression of VEGF in a cell-free system for screening test are added to the VEGF-producing cells and the cells are cultured to determine whether the production of VEGF from said cultured cells is inhibited or not. The compounds whose effect were confirmed in this cultured cell system are further examined for their effect further in experimental animals.

(1) Construction of a plasmid

Before the gene coding for VEGF is sequenced, a plasmid capable of producing VEGF via transcription and translation in a cell-free system is constructed.

The preparation of a plasmid containing the structural gene of VEGF is not particularly limited. For example, it can be constructed as follows:

The upstream and downstream regions of a luciferase structural gene contained in plasmid pPoly(A)-luc(SP6) (Promega) are cleaved off respectively with restriction enzymes ApaI and SacI.

Separately, the VEGF structural gene can be prepared through cloning or isolated from e.g. a plasmid having said gene obtained through cloning. The VEGF structural gene may be derived from humans, bovines, guinea pigs, rats and mice, preferably humans.

The ApaI and SacI sites, which are respectively upstream and downstream of the VEGF structural gene, are cleaved with restriction enzymes ApaI and SacI, respectively. If said restriction sites are not present in the upstream and downstream regions of the VEGF structural gene thus obtained, then a DNA fragment containing said sites can be attached to the gene in a usual manner (J. Sambrook, E. F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd Ed., Gold Spring Harbor Laboratory Press, 1989).

The resulting fragment containing the VEGF structural gene is ligated to the above fragment of plasmid pPoly(A)-luc(SP6) from which the luciferase structural gene was removed with the 2 restriction enzymes. This ligation can be car-

ried out using e.g. a DNA ligation kit available from Takara Shuzo Co., Ltd. The plasmid thus obtained may be introduced into E. coli etc. where the plasmid is replicated in a large amount as the E. coli is multiplied. E. coli JM109 available from Takara Shuzo C ., Ltd. can be used for this purpose.

Then, the desired plasmid is extracted from the E. coli in a usual manner for example as follows:

The cells of E. coli etc. containing the plasmid are harvested by centrifugation. Then, a GTE solution (50 mM glucose, 25 mM Tris-HCl, and 10 mM EDTA, pH 8.0) is added to the cells to obtain cell-suspension, and the cells are lyzed with a mixture of a 1/9 volume of lysozyme solution (50 mg/ml) and a 20/9 volume of sodium hydroxide (0.2 N)-sodium dodecyl sulfate (1 %) relative to the volume of the suspension. The solution is neutralized with potassium acetate (pH 5.2) (final concentration: 1 M), and the insolubles present are removed by centrifugation. To remove the protein present, a mixture of phenol-chloroform-isoamyl alcohol (25 : 24 : 1) is added, and mixed with the sample. After centrifugation upper layer (aqueous layer) is transferred to a new tube, mixed with an equal volume of 2-propanol, and left for a while at room temperature. The sample is centrifuged to give a pellet containing the desired plasmid. The pellet is dissolved in a suitable amount of TE (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), then mixed with solution for cesium chloride density-gradient centrifugation, and separated by ultracentrifugation. The cesium chloride can be removed by dialysis against TE (see Molecular Cloning supra).

The molecular weight of the plasmid thus obtained can be determined by agarose gel electrophoresis, polyacrylamide gel electrophoresis, pulsed field gel electrophoresis, gel filtration chromatography, sedimentation velocity method, light scattering method etc. The nucleotide sequence coding for VEGF can be determined by the Sanger method or Maxam-Gilbert method.

(2) Synthesis of nucleic acid compound

Then, nucleotide sequences each consisting of 8 to 30 contiguous nucleotides whose positions are 1 to 30 nucleotides apart from one another on the nucleotide sequence determined in (1) above are selected, and nucleic acid compounds complementary to the selected nucleotide sequences are synthesized. In a preferable method, nucleotide sequences each consisting of 10 to 20 contiguous nucleotides whose positions are 1 to 13 nucleotides apart from one other on the whole region of the nucleotide sequence coding for VEGF are selected, and nucleic acid compounds complementary to the selected nucleotide sequences are prepared by synthesis.

The nucleic acid compounds include natural-type oligodeoxyribonucleotides, phosphorothioate-type oligodeoxyribonucleotides, phosphorodithioate-type oligodeoxyribonucleotides, methylphosphonate-type oligodeoxyribonucleotides, phosphoramidate-type oligodeoxyribonucle otides, H-phosphonate-type oligodeoxyribonucleotides, triester-type oligodeoxyribonucleotides, α-anomer-type oligodeoxyribonucleotides, those oligoribonucleotide which correspond to said oligodeoxyribonucleotides, peptide nucleic acids, other artificial nucleic acids, and nucleic acid-modified compounds. Among these, the natural-type and phosphorothioate-type oligodeoxyribonucleotides are preferable because of their less nonspecific inhibition of expression, easiness of their synthesis etc. and because their hybrid (double-stranded chain) with mRNA can act as a substrate for RNase H.

The synthesis of the natural-type nucleic compound can be carried out with e.g. a 381A DNA synthesizer or 394 DNA/RNA synthesizer manufactured by ABI (Applied Biosystems Inc.) in accordance with the phosphoramidite method (see instructions available from ABI, or F. Eckstein, Oligonucleotides and Analogues: A Practical Approach, IRL Press (1991)).

In the phosphoramidite method, a nucleic acid-related compound is synthesized by condensation between the 3'-terminal of a modified deoxyribonucleoside or modified ribonucleoside and the 5'-terminal of another modified deoxyribonucleoside, modified ribonucleoside, oligo-modified deoxyribonucleotide or oligo-modified-ribonucleotide by use of a reagent containing phosphoramidite protected with a cyanoethyl group etc.

The final cycle of this synthesis is finished to give a product with a protective group (dimethoxytrityl group etc.) bound to a hydroxyl group at the 5'-terminal of the sugar moiety. The oligomer thus synthesized at room temperature is cleaved off from the support, and its nucleotide and phosphate moieties are deprotected. In this manner, the natural-type oligonucleic acid compound is obtained in a crude form.

The phosphorothioate-type nucleic acid compound can also be synthesized in a similar manner to the above natural type by the phosphoramidite method with the synthesizer from ABI. The procedure after the final cycle of the synthesis is also the same as with the natural type.

The crude nucleic acid compound thus obtained can be purified in a usual manner e.g. ethanol precipitation, or reverse phase chromatography, ion-exchange chromatography and gel filtration chromatography in high performance liquid chromatography (HPLC), supercritical fluid chromatography, and it may be further purified by electrophoresis. A cartridge for reverse phase chromatography, such as tC18-packed SepPak Plus (long body/ENV) (Waters), can also be used.

The purification of the phosphorothioate-type nucleic acid compound (about 3 mg 20-nucleotides compound in a crude form) is carried out in a similar manner to the above natural type.

The purity of the natural-type and phosphorothioate-type nucleic acid compounds can be analyzed by HPLC.

The synthesized nucleic acid compound is used in screening as described below.

(3) Screening

The screening of the nucleic acid compound can be conducted by adding the nucleic acid compound synthesized in (2) above to a transcription and translation assay system in the presence of the VEGF-coding gene or to a translation system in the presence of mRNA transcribed from said gene to examine its inhibition on the expression of VEGF in the system.

This reaction is carried out usually at a temperature of 25 to 40 °C over a period of 0.5 to 3 hours, preferably at 30°C for 1 to 2 hours or at 37°C for 1 hour.

Hereinafter, a method for expressing the protein (VEGF) is described below using a plasmid having the VEGF-coding gene.

Any expression vector containing the VEGF structural gene and being capable of expressing said gene can be used in a usual manner. The transcription and translation system permitting the VEGF structural gene to be expressed for production of VEGF includes that transcription and translation system which is derived from a rabbit reticulocyte lysate or wheat germ extract.

Preferably the expression system makes use of plasmid pSU02. In this case, a transcription and translation system derived from a rabbit reticulocyte lysate, available from Promega, can be used. A kit of TNT™ SP6 Coupled Reticulocyte Lysate System available from Promega is suitable in the case of transcription and translation with pSU02, because an SP6 promoter is located upstream to the VEGF structural gene in plasmid pSU02. The experiment can be conducted according to the manufacture's instructions attached to this kit.

To confirm the formation of VEGF in this transcription and translation system, two methods may be used.

The first method is sandwich-type enzyme linked immunoassays (see e.g. E. Harlow, D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory (1988)) using anti-VEGF polyclonal antibodies obtained from a rabbit which was previously administered with human-derived VEGF and produced in E.coli (see S. Kondo et al., Biochemical and Biophysical Research Communications, Vol. 194, No. 3, pp. 1234 - 1241 (1993)).

This generally well-known method is carried out as follows:

The anti-VEGF polyclonal antibodies obtained as described above are immobilized onto a microtiter plate in a usual manner (see e.g. E. Harlow and D. Lane, Antibodies: A Laboratory Manual, supra). The VEGF-coding gene is added to a transcription and translation assay system and incubated at a suitable temperature for a suitable period. This reaction mixture is put to each well of the microtiter plate. The plate was left at room temperature and then washed. Other anti-VEGF polyclonal antibodies previously labeled with horseradish peroxidase are put to each well, left at room temperature, and washed. An ortho-diaminobenzene solution is added thereto as substrate and left at room temperature until suitable coloration takes place. The absorbance of the solution is determined to estimate the content of VEGF.

The second method makes use of SDS-PAGE (sodium dodecyl sulfatepolyacrylamide gel electrophoresis) and autoradiography to confirm the formation of VEGF in said transcription and translation system.

SDS-PAGE may be carried out in a usual manner (see e.g. the manufacturer's instructions attached to the transcription and translation system kit available from Promega; and Toshio Takagi: "PAGE Polyacrylamide Gel Denkiei-doho" (PAGE Polyacrylamide Gel Electrophoresis), published by Hirokawa Shoten K.K. (1990)). A typical example is as follows.

A 2-mercaptoethanol-containing SDS sample buffer in accordance with the instructions of Promega is added to the reaction mixture in the transcription and translation system, then sealed and thermally treated to denature the protein present. This sample is put to each well on a sodium dodecyl sulfate-polyacrylamide gel attached to an electrophoresis chamber and electrophoresed in the gel (15 % or 17.5 % polyacrylamide gel). To conduct autoradiography, the gel is transferred to a filter paper, dried in an oven, laid on X-ray film in the dark to be introduced into a cassette and left at room temperature over a period of a few hours to tens of hours until the X-ray film is exposed and then the film is developed. If the VEGF-coding gene was expressed, a band appears at a position corresponding to the molecular weight of VEGF. If the expression of said gene was inhibited by the antisense nucleic acid compound, said band does not appear or weakly appears. The position of said band varies depending on the difference of the gene contained in the plasmid used. For example, the band appears at a molecular weight of about 15 kd if pSU02 was used as plasmid, while the band appears at a molecular weight of about 60 kd if pPoly(A)-luc(PS6) was used as plasmid.

RNase H can also be added to the system to improve the inhibitory effect of the antisense nucleic acid compound on the expression of the VEGF-coding gene. RNase H is an enzyme which cleaves mRNA, produced by transcription of DNA, at sites where mRNA forms double strands via hydrogen bond with DNA having complementary nucleotide sequence with mRNA. (H. Stein and P. Hausen, Science, Vol. 166, pp. 393 to 395 (1969); P. Hausen and H. Stein, European Journal of Biochemistry, Vol. 14, pp. 278 to 283 (1970)). By the action of this enzyme, the production of the protein encoded by this gene is inhibited more reliably.

If the natural-type oligodeoxyribonucleotide is used, the concentration of the antisense nucleic acid compound ranges from 0.1 to 10 μM, preferably 0.4 to 2 μM. More preferably the concentration is 0.4 μM in the coexistence of

RNaseH. If the phosphorothioate-type oligodeoxyribonucleotide is used, the concentration of the antisense nucleic acid compound ranges from 0.01 to 1 μM, preferably 0.02 to 0.4 μM. More preferably the concentration is 0.064 to 0.15 μM in the coexistence of RNaseH.

The inhibitory effect of the added nucleic acid compound (i.e. antisense nucleic acid compound) on the expression of the VEGF-coding gene can be evaluated by making a comparison with the expression in the absence of the compound. Specifically, the produced VEGF can be subjected to e.g. SDS-PAGE and then autoradiography as described above to determine the density with a densitometer, where the density in the presence of the antisense nucleic acid compound is compared with the density in the absence of the compound.

The degree of inhibition of the expression of the gene can thus be determined.

To inhibit expression of the gene, the nucleic acid compound has a nucleotide sequence complementary to at least 8 nucleotides, preferably contiguous 8 nucleotides, more preferably at least 14 nucleotides. If one mismatched nucleotide is contained, the nucleic acid compound to inhibit expression of the gene has at least 8 complementary nucleotides, preferably has at least 5 contiguous nucleotide sequence as its shorter contiguous sequence interrupted by said mismatched nucleotide and also has 11 or more complementary nucleotides in total. The number of nucleotides is not limited within these ranges. Although a nucleotide sequence complementary to 30 or more nucleotides may be used, no further improvement can be achieved and it is difficult to synthesize such a long sequence, so the nucleic acid compound having a nucleotide sequence complementary to 30 or less nucleotides suffices to achieve the object of the present invention.

The antisense nucleic acid compound thus obtained is useful as a therapeutic agent to inhibit the growth of solid tumor cells or treat rheumatoid arthritis and diabetes, or as a diagnostic agent for cancers and other diseases.

If the antisense nucleic acid compound of the present invention is used as a therapeutic agent, the object of administration is not particularly limited. For example, the object of administration may be to prevent or treat various kinds of cancer. The compound may be administered orally or parenterally. The oral administration includes sublingual administration. The parenteral administration includes injection (e.g. intracutaneous, intramuscular, intravenous, intraarterial injection), infusion, suppository, ointment, poultice etc. The dose can be varied within a wide range depending on the subject (animal or human), age, administration route, intervals of administration. The dose of the effective antisense nucleic acid compound itself presented in the present invention or the dose of the antisense nucleic acid compound used with a suitable diluent or with a pharmacologically acceptable carrier ranges from 1 to 80,000 μg/kg body weight/day, and is administered successively or once or in portions per day.

Tablets, granules, powder, capsules etc., in the case of oral administration of the antisense nucleic acid compound of the invention, contain customary additives such as binders, fillers, lubricants, disintegrator, wetting agent etc. Liquid preparations for oral administration may be in the form of oral aqueous agent, suspension, emulsion, syrup etc. A dried product may also be used which is dissolved before use. The composition may further contain any additives and preservatives.

In the case of parenteral administration, the preparation contains additives such as stabilizer, buffer, preservative, isotonic agent etc., usually in an ampoule for single administration, a vessel for multiple administrations or in a tube. The composition may be powder to be dissolved in suitable liquid e.g. pyrogen-free sterilized water.

The antisense nucleic acid compound of the invention can be used as a diagnostic agent for e.g. cancer. Because it is known that cancer cells generally produce VEGF, cancer cells can be diagnosed by examining the degree of expression of VEGF in cells with a probe that is the nucleic acid compound of the invention having a nucleotide sequence complementary to a specific nucleotide sequence in the VEGF-coding gene or its transcribed mRNA.

(4) Effect of the antisense nucleic acid in cultured cell and experimental animal assay systems

A pharmacological test is carried out in the following manner to evidence that the antisense nucleic acid compound of the invention is useful as a therapeutic or diagnostic agent.

A natural-type oligodeoxyribonucleotide or phosphorothioate-type oligodeoxyribonucleotide expected to have a nucleotide sequence with the antisense nucleic acid effect is used as the antisense nucleic acid compound to evaluate its effect on the expression of VEGF in a cultured cell system, as follows: The antisense nucleic acid compound such as natural-type oligodeoxyribonucleotide or phosphorothioate-type oligodeoxyribonucleotide is added at a concentration of 0.01 to 100 μM, preferably 0.1 to 10 μM, to cells derived mammals such as human, mouse, rat, guinea pig, bovine etc. under germ-free conditions, if necessary in the presence of reagents facilitating incorporation of antisense nucleic acid compound into cells, such as a lipofectin reagent, lipofectamine reagent, DOTAP reagent, artificial synthetic lipid vehicle, liposome$_1$ membrane fusion reagent, polymeric micellar reagent, polymeric carrier etc. The inhibition of the expression of the target protein (VEGF) can then be evaluated by e.g. ELISA (enzyme-linked immunoassay) or Western blotting using anti-VEGF antibody to confirm the inhibitory effect of the antisense nucleic acid compound on the expression of the protein.

A natural-type oligodeoxyribonucleotide or phosphorothioate-type oligodeoxyribonucleotide having a nucleotide sequence of expected antisense effect is used as the antisense nucleic acid compound to evaluate its effect on the

expression of VEGF in an experimental animal system, as follows: The antisense nucleic acid compound such as natural-type oligodeoxyribonucleotide or phosphorothioate-type oligodeoxyribonucleotide is administered at a dose of 0.001 to 100 mg/kg body weight, preferably 0.1 to 80 mg/kg body weight, into mammals such as mouse, rat, guinea pig, rabbit etc., if necessary together with drug delivery system reagents, such as a lipofectin reagent, lipofectamine reagent, DOTAP reagent, artificial synthetic lipid vehicle, liposome, membrane fusion reagent, polymeric micellar reagent, polymeric carrier etc. via intravenous injection, intra-arterial injection, intracutaneous injection, intraperitoneal administration, or topical administration. The inhibition of the expression of the target protein (VEGF) can then be evaluated by e.g. ELISA or Western blotting using anti-VEGF antibody to confirm the inhibitory effect of the antisense nucleic acid on the expression of the protein. Further, human-derived cancer cells etc. can also be transplanted to animals such as mouse, rat, guinea pig and rabbit to evaluate the effect of the antisense nucleic acid compound such as natural-type oligodeoxyribonucleotide or phosphorothioate-type oligodeoxyribonucleotide on the growth of said cells. In this case, too, the antisense nucleic acid compound is administered at a dose of 0.001 to 100 mg/kg body weight, preferably 0.1 to 80 mg/kg body weight into the animals, if necessary together with drug delivery system reagents, such as a lipofectin reagent, lipofactamine reagent, DOTAP reagent, artificial synthetic lipid vehicle, liposome, membrane fusion reagent, polymeric micellar reagent, polymeric carrier etc. via intravenous injection, intra-arterial injection, intracutaneous injection, intraperitoneal administration, or topical administration. On the basis of the degree of inhibition of cancer cell growth by the antisense nucleic acid compound and the number of survival days of the experimental animals, the antisense nucleic acid effect can be evaluated.

Specific examples of such tests will be described below.

Description of the Drawings

FIG. 1 shows the result of agarose gel electrophoresis for the plasmid (pSU02). In this photograph, lane 1 shows pSU02 only; lane 2, pUS02 treated with restriction enzyme ApaI (10 U); and lane 3, pUS02 treated with restriction enzyme ApaI (20 U). M shows a molecular weight marker.

FIG. 2 shows a result of HPLC for a natural-type oligodeoxyribonucleotide.

FIG. 3 shows a result of HPLC for a phosphorothioate-type oligodeoxyribonucleotide.

FIG. 4 shows an UV absorption spectrum of a natural-type oligodeoxyribonucleotide.

FIG. 5 shows an UV absorption spectrum of a phosphorothioate-type oligodeoxyribonucleotide.

FIG. 6 shows the amount of VEGF expressed as determined by enzyme immunoassays.

FIG. 7 shows results of SDS-PAGE for VEGF and luciferase formed in a transcription and translation system. Lanes 1 and 3 show a band of VEGF expressed using plasmid pSU02, and lanes 2 and 4 show a band of luciferase expressed using plasmid pPoly(A)-luc(SP6).

FIG. 8 shows SDS-PAGE analysis for the effect of RNase H on the activity of a transcription and translation system derived from rabbit reticulocyte lysate (lane 1 in the absence of RNase H, and lanes 2, 3 and 4 in the presence of 1, 5 and 25 U RNase H, respectively).

FIG. 9 shows activity of RNase H in a transcription and translation system.

FIG. 10 shows the effect of the nucleic acid compound on the expression of VEGF ("■" in the presence of RNase H and "□" in the absence of RNase H).

FIG. 11 shows the effect of the nucleic acid compound on the expression of VEGF ("■" in the presence of RNase H and "□" in the absence of RNase H).

FIG. 12 shows the effect of the nucleic acid compound on the expression of VEGF ("-●-" with the nucleic acid compound A101 in the presence of 11.4 U RNase H, "-○-" with A101 in the absence of RNase H, "-▲-" with A143 in the presence of 11.4 U RNase H, "-△-" with A143 in the absence of RNase H, and "-■-" with RA101 in the presence of 11.4 U RNase H).

Best Mode for Carrying Out the Invention

The present invention is described in more detail by referring to the following examples, which however are not intended to limit the scope of the present invention.

[Example 1]

(1) Construction and sequencing of the plasmid

The upstream and downstream regions of a luciferase structural gene contained in plasmid pPoly(A)-luc(SP6) (Promega) were cleaved off with restriction enzymes ApaI and SacI, respectively. Separately, a gene coding for human-derived VEGF obtained by the PCR method was inserted into a region between ApaI and XbaI sites in a multicloning

site in plasmid pRC/CMv, whereby plasmid pSU01 was constructed, and then its upstream ApaI site and downstream SacI site were cleaved with restriction enzymes ApaI and SacI, respectively.

Using a DNA ligation kit available from Takara Shuzo Co., Ltd., the above ApaI- and SacI-cleaved fragment containing the VEGF structural gene was ligated to the above pPoly(A)-luc(SP6) fragment from which the luciferase structural gene had been removed with the 2 restriction enzymes ApaI and SacI (the ligation method followed the manufacture's instructions).

Then, the plasmid thus obtained was introduced into E. coli competent cell JM109 available from Takara Shuzo Co., Ltd. according to the manufacture's instructions and then replicated in a large amount. The E. coli cells carrying the plasmid were harvested by centrifugation. A GTE solution (50 mM glucose, 25 mM Tris-HCl, and 10 mM EDTA, pH 8.0) was added to the cells to prepare a suspension, and the cells were lyzed with a mixture of a 1/9 volume of lysozyme solution (50 mg/ml) and a 20/9 volume of sodium hydroxide (0.2 N)-sodium dodecyl sulfate (1 %) relative to the volume of the suspension. The solution was neutralized with potassium acetate (pH 5.2) (final concentration: 1 M), and the insolubles present were removed by centrifugation. To remove the protein present, a mixture of phenol-chloroform-isoamyl alcohol (25 : 24 : 1) was added and mixed with the sample. After centrifugation, the upper layer (aqueous layer) was transfered to a new tube, mixed with an equal volume of 2-propanol, and left for a while at room temperature. The sample was centrifuged to give a pellet containing the desired plasmid (designated pSU02). The pellet was dissolved in a suitable amount of TE (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), then mixed with solution for cesium chloride density-gradient centrifugation, and separated by ultracentrifugation. The resulting plasmid was dialyzed against TE to remove the cesium chloride (see Molecular Cloning supra). The desired plasmid (pSU02), 0.7 mg, was thus obtained.

Agarose gel electrophoresis indicated that this plasmid (pSU02) was about 3.6 kbp long (FIG. 1). In FIG. 1, lane 1 shows pSU02 only; lane 2, pUS02 treated with restriction enzyme ApaI (10 U); and lane 3, pUS02 treated with restriction enzyme ApaI (20 U). M shows a molecular weight marker.

Then, the nucleotide sequence of the VEGF structural gene including its surrounding regions in plasmid pSU02 was determined by the Sanger method. The nucleotide sequence thus determined is shown in SEQ ID NO:1.

(2) Synthesis and purification of natural-type and phosphorothioate-type nucleic acid compounds

Nucleotides whose sequences were complementary to 20 contiguous nucleotides that are 6 nucleotide apart from one another between the 77-position and the 570-position in SEQ ID NO:1 were synthesized in 381A DNA synthesizer or 394 DNA/RNA synthesizer manufactured by ABI by the phosphoramidite method in accordance with the manufacture's instructions. Eighty nucleotides thas obtained are shown in Table 1, i.e. A077 to A551. Five nucleic acid compounds each consisting of 20 nucleotides, i.e. S101 to T-20 shown in Table 1 and 77 nucleic acid compounds each consisting of 6 to 18 nucleotides in Table 2 were also synthesized in a similar manner. The final cycle of this synthesis was finished to give a product with a protective group (dimethoxytrityl group) bound to the 5'-terminal hydroxyl group of its sugar moiety. At room temperature, the synthesized oligomer was cleaved off from the support by treatment with about 25 % ammonium water for 60 minutes. The product was kept at 55 °C for 8 hours to deprotect its base and phosphate moieties.

In this manner, the natural-type oligodeoxyribonucleotides were obtained in crude form.

Each of the crude natural-type oligodeoxyribonucleotides was purified through a cartridge, SepPak Plus (long body/ENV packed with tC18, manufactured by Waters) for reverse phase chromatography, as follows:

The inside of the cartridge was washed with 20 ml acetonitrile and then equilibrated with 20 ml of 12 % acetonitrile-88 % TEAA (TEAA: 0.1 M triethyl ammonium acetate, pH 7.2). The crude oligodeoxyribonucleotide was dissolved in about 3 ml of 12 % acetonitrile-88 % TEAA and injected into the cartridge, and the elute flowed out in this injection was returned to the cartridge by injection, and the same procedure was repeated again. After the cartridge was washed with 15 ml of 12 % acetonitrile-88 % TEAA, the solution in the cartridge was replaced by 3 ml TEAA. Then, 3 ml of 2 % aqueous trifluoroacetic acid was injected into the cartridge and left for about 4 minutes to cleave off the dimethoxytrityl group. Additional 3 ml of 2 % aqueous trifluoroacetic acid was injected to push out the previous aqueous trifluoroacetic acid from the cartridge. The inside of the cartridge was replaced by 3 ml TEAB (triethylammonium bicarbonate, pH 7) and the sample was eluted with 8 ml of 15 % acetonitrile-85 % TEAB. The fractions containing the purified oligodeoxyribonucleotide were collected and evaporated into dryness under reduced pressure. 0.2 ml sterilized physiological saline was added to this sample, and it was evaporated into dryness again under reduced pressure. A small amount of sterilized water was added to this sample and it was evaporated again, and this procedure was repeated. Then, the same amount of sterilized water as the initially added physiological saline was added to the sample, and it was diluted to a predetermined concentration (500 μM oligodeoxyribonucleotide) and used in the screening experiment described below. For evaluation of the amount of the nucleic acid compound, it was assumed that 33 μg oligodeoxyribonucleotide in 1 ml buffer (20 mM sodium phosphate buffer, 100 mM NaCl, pH 7.0) has an absorbance of 1 at 260 pm as determined at room temperature in a cuvette of 1 cm light path length, and that the molecular weight of the natural-type oligodeoxyribonucleotide per nucleotide is 330. Alternatively, the amount of the nucleic acid compound was determined at 70 to 80°C according to the nearest-neighbor approximation method on the basis of the reported molecular absorption coef-

ficients of mononucleotide and dinucleotide (E. G. Richards, Handbook of Biochemistry and Molecular Biology: Nucleic Acids (edited by C. D. Fasman), 3rd ed., vol. I, p. 197, CRC Cleveland, OH). The concentrations of the nucleic acid compounds described in the present specification were determined in any of the above methods.

The purification of the crude phosphorothioate-type oligodeoxyribonucleotide (about 3 mg crude product of 20 nucleotides) was also carried out in a similar manner to the above natural type except that 20 % acetonitrile-80 % TEAA (or TEAB) was used in equilibrating the cartridge, washing the cartridge after application of the crude sample, and eluting the purified phosphorothioate-type oligodeoxyribonucleotide.

The nucleotide sequences of the natural-type deoxyribonucleotides synthesized are shown in Tables 1 and 2.

Table 1

sample #	nucleotide sequence 5' — — 3'	expression (%)
A077	TCGGAGGCCCGACCGGGGCC	>100
A083	ATGGTTTCGGAGGCCCGACC	37
A089	AAGTTCATGGTTTCGGAGGC	6
A095	AGCAGAAAGTTCATGGTTTC	6
A101	CAAGACAGCAGAAAGTTCAT	25
A107	TGCACCCAAGACAGCAGAAA	16
A113	CTCCAATGCACCCAAGACAG	16
A119	GCAAGGCTCCAATGCACCCA	14
A125	AGCAAGGCAAGGCTCCAATG	18
A131	TAGAGCAGCAAGGCAAGGCT	28
A137	TGGAGGTAGAGCAGCAAGGC	21
A143	GCATGGTGGAGGTAGAGCAG	0
A149	CACTTGGCATGGTGGAGGTA	4
A155	TGGGACCACTTGGCATGGTG	9
A161	GCAGCCTGGGACCACTTGGC	3
A167	ATGGGTGCAGCCTGGGACCA	37
A173	TCTGCCATGGGTGCAGCCTG	67
A179	CCTCCTTCTGCCATGGGTGC	5
A185	TGCCCTCCTCCTTCTGCCAT	5
A191	TGATTCTGCCCTCCTCCTTC	7
A197	TCGTGATGATTCTGCCCTCC	0
A203	ACCACTTCGTGATGATTCTG	13
A209	AACTTCACCACTTCGTGATG	29
A215	TCCATGAACTTCACCACTTC	11
A221	TAGACATCCATGAACTTCAC	18
A227	CGCTGATAGACATCCATGAA	3
A233	TAGCTGCGCTGATAGACATC	23
A239	TGGCAGTAGCTGCGCTGATA	43
A245	ATTGGATGGCAGTAGCTGCG	50
A251	GTCTCGATTGGATGGCAGTA	0
A257	ACCAGGGTCTCGATTGGATG	3
A263	ATGTCCACCAGGGTCTCGAT	1
A269	TGGAAGATGTCCACCAGGGT	46
A275	TACTCCTGGAAGATGTCCAC	16
A281	TCAGGGTACTCCTGGAAGAT	1
A287	ATCTCATCAGGGTACTCCTG	1
A293	TACTCGATCTCATCAGGGTA	12
A299	AAGATGTACTCGATCTCATC	0
A305	GGCTTGAAGATGTACTCGAT	0
A311	CAGGATGGCTTGAAGATGTA	4
A317	GGCACACAGGATGGCTTGAA	11
A323	ATCAGGGGCACACAGGATGG	23
A329	CATCGCATCAGGGGCACACA	51
A335	CCCCCGCATCGCATCAGGGG	58
A341	CAGCAGCCCCCGCATCGCAT	39
A347	TCATTGCAGCAGCCCCCGCA	9
A353	CCCTCGTCATTGCAGCAGCC	1
A359	TCCAGGCCCTCGTCATTGCA	0
A365	ACACACTCCAGGCCCTCGTC	14
A371	GTGGGCACACACTCCAGGCC	35

sample #	nucleotide sequence 5' — — 3'	expression (%)
A377	TCCTCAGTGGGCACACACTC	28
A383	TTGGACTCCTCAGTGGGCAC	6
A389	GTGATGTTGGACTCCTCAGT	0
A395	TGCATGGTGATGTTGGACTC	0
A401	ATAATCTGCATGGTGATGTT	0
A407	ATCCGCATAATCTGCATGGT	0
A413	GGTTTGATCCGCATAATCTG	0
A419	TGGTGAGGTTTGATCCGCAT	0
A425	TGGCCTTGGTGAGGTTTGAT	0
A431	ATGTGCTGGCCTTGGTGAGG	0
A437	TCTCCTATGTGCTGGCCTTG	0
A443	CTCATCTCTCCTATGTGCTG	0
A449	AGGAAGCTCATCTCTCCTAT	0
A455	TGCTGTAGGAAGCTCATCTC	0
A461	TTGTTGTGCTGTAGGAAGCT	0
A467	TCACATTTGTTGTGCTGTAG	3
A473	CTGCATTCACATTTGTTGTG	0
A479	TTTGGTCTGCATTCACATTT	0
A485	TCTTTCTTTGGTCTGCATTC	0
A491	GCTCTATCTTTCTTTGGTCT	0
A497	TGTCTTGCTCTATCTTTCTT	0
A503	TTTTCTTGTCTTGCTCTATC	0
A509	TCACATTTTTCTTGTCTTGC	0
A515	GGCTTGTCACATTTTTCTTG	0
A521	CGCCTCGGCTTGTCACATTT	0
A527	GCTCACCGCCTCGGCTTGTC	37
A533	TGCCCGGCTCACCGCCTCGG	64
A539	TCCTCCTGCCCGGCTCACCG	11
A545	GCTCCTTCCTCCTGCCCGGC	0
A551	AGGGAGGCTCCTTCCTCCTG	68
S101	ATGAACTTTCTGCTGTCTTG	44
RA101	AACTATAAGCACGGTAACGA	66
RA143	GAAGTGAGCGTGAGCGTGAG	57
RS143	CTCACGCTCACGCTCACTTC	38
T-20	TTTTTTTTTTTTTTTTTTTT	70

Table 2

sample #	nucleotide sequence 5' — —3'	expression (%)
A083N	TCGGAGGCCCGACC	46
A085N	TTTCGGAGGCCCGA	37
A085R	ATGGTTTCGGAGGCCCGA	6
A087P	ATGGTTTCGGAGGCCC	7
A089N	ATGGTTTCGGAGGC	1
A095N	AAGTTCATGGTTTC	0
A101N	AGCAGAAAGTTCAT	8
A105N	AGACAGCAGAAAGT	18
A108N	CCAAGACAGCAGAA	23
A109N	CCCAAGACAGCAGA	18
A110N	ACCCAAGACAGCAG	25
A143N	TGGAGGTAGAGCAG	17
A146N	TGGTGGAGGTAGAG	5
A153N	CTTGGCATGGTGGA	1
A155N	CACTTGGCATGGTG	3
A156N	CCACTTGGCATGGT	5
A157N	ACCACTTGGCATGG	17
A167N	GCAGCCTGGGACCA	2
A173N	ATGGGTGCAGCCTG	29
A176N	GCCATGGGTGCAGC	12
A179N	TCTGCCATGGGTGC	1
A189N	CCCTCCTCCTTCTG	0
A191N	TGCCCTCCTCCTTC	1
A193N	TCTGCCCTCCTCCT	0
A203N	TCGTGATGATTCTG	0
A209N	ACCACTTCGTGATG	11
A213N	CTTCACCACTTCGT	2
A217N	TGAACTTCACCACT	4
A237N	GCTGCGCTGATAGA	2
A242N	CAGTAGCTGCGCTG	11
A245N	TGGCAGTAGCTGCG	19
A248N	GGATGGCAGTAGCT	2
A251N	ATTGGATGGCAGTA	0
A261N	CAGGGTCTCGATTG	0
A263N	ACCAGGGTCTCGAT	0
A265N	CCACCAGGGTCTCG	10
A275N	TGGAAGATGTCCAC	85

sample #	nucleotide sequence 5' — —3'	expression (%)
A293N	ATCTCATCAGGGTA	8
A296N	TCGATCTCATCAGG	9
A299N	TACTCGATCTCATC	9
A303N	GATGTACTCGATCT	1
A313N	ATGGCTTGAAGATG	3
A317N	CAGGATGGCTTGAA	4
A319N	CACAGGATGGCTTG	21
A321N	CACACAGGATGGCT	9
A323N	GGCACACAGGATGG	38
A325N	GGGGCACACAGGAT	51
A347N	CAGCAGCCCCCGCA	37
A351N	ATTGCAGCAGCCCC	24
A356N	TCGTCATTGCAGCA	0
A361N	GGCCCTCGTCATTG	0
A365N	TCCAGGCCCTCGTC	2
A368N	CACTCCAGGCCCTC	33
A371N	ACACACTCCAGGCC	41
A379R	TCCTCAGTGGGCACACAC	45
A381P	TCCTCAGTGGGCACAC	35
A383N	TCCTCAGTGGGCAC	4
A397N	TGGTGATGTTGGAC	0
A422N	TGAGGTTTGATCCG	1
A423L	GAGGTTTGATCC	1
A424J	AGGTTTGATC	1
A425H	GGTTTGAT	53
A426F	GTTTGA	78
A473N	TCACATTTGTTGTG	3
A473L	ACATTTGTTGTG	0
A473J	ATTTGTTGTG	1
A473I	TTTGTTGTG	49
A473H	TTGTTGTG	33
A473F	GTTGTG	34
A497N	GCTCTATCTTTCTT	1
A499L	GCTCTATCTTTC	1
A501J	GCTCTATCTT	1
A503H	GCTCTATC	1
A505F	GCTCTA	75
A513N	ACATTTTTCTTGTC	0
A521N	GGCTTGTCACATTT	1
A527N	CGCCTCGGCTTGTC	35

In item "sample #" in Table 1, the nucleotide sequences given "A" before their numbers are antisense chains, i.e. chains complementary to a partial sense chain in the nucleic acid sequence coding for VEGF, and the nucleotide sequences given "S" are sense chains. The nucleotide sequences given "R" are random sequences; for example, the nucleotide sequence "RA101" is identical in nucleotide composition with the nucleotide "A101", but each of its nucleotides is randomly positioned.

The number given for each nucleotide sequence indicates the beginning position of its corresponding nucleotide sequence in SEQ ID NO:1. For example, the nucleotide sequence "A101" is an antisense nucleic acid compound of 20-nucleotides, which consists of a nucleotide sequence complementary to the nucleotide sequence of from the 101- to 120-positions in SEQ ID NO:1. The specific sequence (20 nucleotides) of each sample # is shown in item "nucleotide sequence" in Table 1. The nucleotide sequence "T-20" is a nucleic acid compound consisting of 20 thymidines.

In item "sample #" in Table 2, the designation "A" has the same meaning as in Table 1. Each alphabet after sample number indicates degree of polymerization as follows: F means 6-nucleotides compound; H, 8-nucleotides; I, 9-nucleotides; J, 10-nucleotides; L, 12-nucleotides; N, 14-nucleotides; P, 16-nucleotides; and R, 18-nucleotides. The specific sequence of each sample# is shown in item "nucleotide sequence".

The natural-type and phosphorothioate-type oligodeoxyribonucleotides were examined for their purity with HPLC and UV absorption spectra. The elution conditions, column etc. are as follows:

(I) HPLC

(a) For the natural-type oligodeoxyribonucleotide

Sample: A101.
Column: Ion-exchange column (Gen-Pak DNA 6×150 mm available from Waters).
Solvent A: Mixture (9 : 1) of 25 mM sodium phosphate buffer, pH 6.0 and acetonitrile.
Solvent B: Solvent A plus 1 M NaCl.
Elution: Linear gradient at 0.8 ml/min. of from 20 % Solvent B (i.e. 80 % Solvent A) to 100 % Solvent B for 40 min.

The result is shown in FIG. 2.

(b) For the phosphorothioate-type oligodeoxyribonucleotide

Sample: Compound S101-S being identical in nucleotide sequence with S101 but having a phosphorothioate-type bond in place of the phosphate diester bond.
Column: Reverse phase column (μ Bondasphere 5 μ C18 300 A, 3.9×150 mm, available from Waters).
Solvent A: 0.1 M aqueous triethyl ammonium acetate (pH 7.0).
Solvent B: Acetonitrile.
Elution: Linear gradient at 1 ml/min. of from 10 % Solvent B (i.e. 90 % Solvent A) to 60 % Solvent B for 25 min., and then a constant concentration of 60 % Solvent B at 1 ml/min. for 5 min.

The result is shown in FIG. 3.

(ii) Analysis of UV absorption spectra

Sample: The same natural- and phosphorothioate-type oligodeoxyribonucleotides as used in HPLC.
Solvent: 20 mM sodium phosphate buffer, pH 7.0, plus 0.1 M NaCl for both the natural- and phosphorothioate-type oligodeoxyribonucleotides.

FIGS. 4 and 5 show the results of the natural- and phosphorothioate-type nucleotides, respectively.

FIGS. 2 and 3 show that the purity of these compounds is sufficient for use in the experiments of the present invention. FIGS. 4 and 5 show that the UV absorption spectra of these compounds agree with those of nucleic acid compounds.

(3) Expression of VEGF

A transcription and translation system derived from a rabbit reticulocyte lysate, available from Promega, can be used for expression of VEGF from plasmid pSU02. Because an SP6 promoter is located upstream to the VEGF structural gene in plasmid pSU02, a kit of TNT ™ SP6 Coupled Reticulocyte Lysate System was used for transcription and translation of pSU02. The experimental method followed the manufacturer's instructions attached to the kit.

Table 3 shows the composition of the reaction mixture in this transcription and translation system.

Table 3

sample	amount (μl)
TNT™ rabbit reticulocyte lysate	12.5
TNT™ reaction buffer	1.0
TNT™ SP6 RNA polymerase	0.5
Amino acid mixture (1 mM; not containing methionine)	0.5
^{35}S-methionine	1.0
Ribonuclease Inhibitor (40 U/μl)	0.5
pSU02 (0.5 μg/μl)	1.0
Sterilized water	8.0
Total	25.0

^{35}S-methionine was of in vivo cell labeling grade (SJ1015, 37TBq/mmol, 0.37 MBq μl) manufactured by Amersham and it was added in a half (1 μl) of the prescribed amount in the instructions attached to the kit of Promega. The ribonuclease inhibitor was supplied from Takara Shuzo Co., Ltd., and the sterilized water was previously treated at 121 °C for 15 minutes. The other ingredients except for pSU02 were those contained in the kit of Promega. The above reaction mixture was incubated at 30°C or 37°C for 1 to 2 hours to produce 10 to 100 ng of the protein of VEGF.

(4) Confirmation of expression of VEGF

(i) Enzyme immunoassays

Anti-VEGF polyclonal antibodies obtained from a rabbit given human-derived VEGF produced by E. coli were immobilized in a usual manner onto a polystyrene microtiter plate. Then, the mixture described in (3) above (previously reacted at 30°C for 2 hours) for the transcription and translation system for VEGF was diluted 3- to 9375-fold and the diluted mixture was added to each well. The plate was left at room temperature (25 °C) for 2 hours. The diluted mixture was removed, and the plate was washed with a phosphate buffer containing 0.1 % bovine serum albumin. Then, other anti-VEGF polyclonal antibodies labeled with horseradish peroxidase were added to each well and left at room temperature for 1 hour. The plate was washed sufficiently with the same phosphate buffer, and an ortho-diaminobenzene solution was added thereto as substrate and left at room temperature until suitable coloration took place (about 30 minutes). Thereafter, the absorbance of each solution was determined at 490 nm to evaluate its VEGF content.

The results are shown in FIG. 6. If pSU02 was used as the plasmid, the absorbance of the protein (VEGF) ("○" in the graph) was evidently higher in low degree of dilution (from 3-fold to 375-fold) than in high degree of dilution (from 1875-fold to 9375-fold) of the reaction mixture. On the other hand, if pPoly(A)-luc(SP6) not producing VEGF was used as the plasmid, the absorbance remained nearly constant ("●" in the graph and overlapped with ◎).

That is, if pPoly(A)-luc(SP6) was used as the plasmid, the absorbance is substantially the same as in high degree of dilution of the reaction mixture as well as in the control ("◎" in the graph) where water was used in place of pSU02.

Hence, it can be concluded that the higher absorbance in the case of low dilution of the pSU02-containing reaction mixture was due to the production of VEGF in the transcription and translation system. Three-fold dilution of the reaction mixture containing pSU02 as the plasmid gave lower absorbance than 15-fold dilution of the same reaction mixture. This suggests that the reaction mixture contained a substance significantly inhibiting the reaction between VEGF and the polyclonal antibodies.

(ii) Electrophoresis

The formation of VEGF in the above transcription and translation system was confirmed in SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

SDS-PAGE was carried out in the following manner according to the instructions attached to the transcription and translation system kit available from Promega.

Five μl of the reaction mixture in the transcription and translation system was added to 20 μl of 2-mercaptoethanol containing SDS sample buffer with the composition in accordance with the instructions of Promega. The mixture was

sealed and heated at 100°C for 2 minutes to denature the protein. Five μl of the mixture was removed and electrophoresed by SDS-PAGE (15 % or 17.5 % polyacrylamide gel). For autoradiography, the gel was transferred to a filter paper and dried sufficiently at 80°C in an oven. Then, the gel was laid on X-ray film in the dark to be introduced into a cassette. This cassette was left at room temperature for 10 to 100 hours, and the X-ray film was developed.

The result is shown in Fig. 7. Lanes 1 and 3 show bands of the reaction mixture where pSU02 was used as the plasmid, and lanes 2 and 4 show bands of the reaction mixture where pPoly(A)-luc(SP6) was used as the plasmid. The reaction mixtures used in lanes 1 and 2 were previously incubated at 30°C for 2 hours in the transcription and translation system, while the reaction mixtures in lanes 3 and 4 were previously incubated at 37 °C for 2 hours in the system. The molecular weight of each band was estimated from the positions of simultaneously electrophoresed pigment-labeled proteins as the molecular-weight marker [Rainbow™ marker (high-molecular range)]. If pSU02 was used as the plasmid, a band with a molecular weight of about 15 kd was observed, but if pPoly(A)-luc(SP6) was used as the plasmid, a band appeared at a molecular weight of about 60 kd in place of said band of about 15 kd. The molecular weight of VEGF produced using plasmid pSU02 estimated to be 17.2 kd from its amino acid sequence, and from this molecular weight it is understood that VEGF was produced from plasmid pSU02.

(5) Screening

On the basis of the results of (1) to (4) above, screening was carried out using the transcription and translation system (Promega) derived from a rabbit reticulocyte lysate.

First, the optimum conditions for screening were examined in the following manner.

(i) Examination of natural-type and phosphorothioate-type oligodeoxyribonucleotides and reaction temperature

Whether the oligodeoxyribonucleotides (natural-type and phosphorothioate-type) obtained in (2) above are suitable for use in screening as the antisense nucleic acid compound was evaluated by examining their stability and effect on the transcription and translation system. The results are shown below.

The natural-type and phosphorothioate-type oligodeoxyribonucleotides each consisting of about 20 nucleotides were labeled at the 5'-terminal with a ^{32}P-phosphate group by use of T4 polynucleotide kinase (Takara Shuzo Co., Ltd.), and their stability was examined in the transcription and translation system (Promega) derived from a rabbit reticulocyte lysate. The result indicated that almost all the natural-type and phosphorothioate-type oligodeoxyribonucleotides remained stable at 30°C for 3 hours or at 37 °C for 2.5 hours. The decomposition of the natural-type and phosphorothioate-type oligodeoxyribonucleotides, particularly at 37°C for 1 hour, was almost negligible.

From this result, it can be concluded that the natural-type and phosphorothioate-type oligodeoxyribonucleotides are suitable for use in screening to examine the effect of antisense nucleic acid compound. Although it was found that the screening temperature in the transcription and translation system (Promega) derived from a rabbit reticulocyte lysate may be 30 °C and 37 °C, the latter was adopted for screening since 37 °C is near the body temperature.

(ii) Examination of the action of RNase H

The effect of RNase H (enzyme cleaving a double-stranded chain of a hybrid between mRNA and the antisense nucleic acid compound) on the transcription and translation system (Promega) used in screening of the antisense nucleic acid compound was examined by SDS-PAGE and autoradiography. The reaction mixture in the transcription and translation system for this purpose is shown in Table 4.

Table 4

sample	amount (μl)
TNT™ rabbit reticulocyte lysate	5.0
TNT™ reaction buffer	0.4
TNT™ SP6 RNA polymerase	0.2
Amino acid mixture (1 mM; not containing methionine)	0.2
^{35}S-methionine	0.8
Ribonuclease Inhibitor (40 U/μl)	0.2
Plasmid pSU02 (0.5 μg/μl)	0.4
RNase H or sterilized water	2.8
Total	10.0

In the above composition, the ingredients other than RNase H were those described in (1) or (3) above. RNase H, available from Takara Shuzo Co., Ltd., was adjusted to 1, 5, or 25 U in total in the reaction mixture. After incubation at 37 °C for 1 hour, the reaction mixture was subjected to SDS-PAGE and autoradiography as described in (4) above. The result is shown in FIG. 8.

This result indicated that the addition of RNase H brought about no significant decrease in the density of the VEGF band ("→" in FIG. 8), and thus it was confirmed that the activity of the transcription and translation system (Promega) was not decreased even by addition of RNase H.

In FIG. 8, lane 1 shows the reaction mixture where RNase H was not added, and lanes 2, 3 and 4 show the reaction mixtures containing 1, 5, and 25 U RNase H, respectively.

It was then examined whether RNaseH activity can appear in the transcription and translation system (Promega) derived from a rabbit reticulocyte lysate. This examination was carried out in the following manner by referring to the method of the literature (I. Berkower et al., Journal of Biological Chemistry, vol. 248, pp. 5914 - 5921 (1974)). The composition of the assay mixture is shown in Table 5.

Table 5

sample	amount (μl)
TNT™ rabbit reticulocyte lysate	12.5
TNT™ reaction buffer	1.0
TNT™ SP6 RNA polymerase	0.5
Amino acid mixture (1 mM; not containing 1 mM methionine)	0.5
Amino acid mixture (1 mM; not containing 1 mM leucine)	0.5
Ribonuclease Inhibitor (40 U/μl)	0.5
Plasmid pSU02 (0.5 μg/μl)	1.0
poly[^{3}H-rA] (0.1 μg/μl)	1.1
poly[dT] (0.1 μg/μl)	1.0
RNase H (1.0 or 10.0 U/μl)	1.0 (or 2.5)
Sterilized water	5.4 (or 3.9)
Total	25.0

The ingredient poly[^{3}H-rA], available from Amersham, had a polymerization degree of 38 to 137 and a radioactivity of 51.8 MBq/mg. The ingredient poly[dT] available from Pharmacia had a mean polymerization degree of 174. The

amino acid mixture (1 mM, not containing leucine) was contained in the transcription and translation system (Promega) derived from a rabbit reticulocyte lysate. The other ingredients were those described in (1) or (3) above.

The above mixture was incubated at 37 °C for 20 minutes, and then the following regents were added.

0.1 M sodium pyrophosphate (4 °C)	50 µl
Salmon sperm DNA (thermally denatured, 1 mg/ml)	25 µl
Bovine serum albumin (10 mg/ml)	50 µl
10 % aqueous trichloroacetic acid	150 µl

The mixture thus obtained was gently stirred and then centrifuged at 4000 r.p.m. for 2 minutes. The supernatant was introduced into a minivial for liquid scintillation, and 2 ml reagent for liquid scintillation (Ultima Gold, manufactured by Packard) was introduced into the minivial. The mixture was gently shaken and examined for radioactivity in a liquid scintillation counter (Beckman). The result indicated that the significant activity of RNase H was present under the conditions of the above reaction (FIG. 9).

The influence of coexistent RNase H on the effect of the oligodeoxyribonucleotide in different concentrations was examined. The experimental method is as follows:

The amount of VEGF expressed in the presence or absence of RNase H, with the nucleic acid compound in a concentration ranging from 0 to 2000 nM, was determined as described above in (4) (ii). For this experiment, RNase H (11.4 U) and the nucleic acid compound (final concentration: 16 to 2000 nM) were added as necessary to the reaction mixture shown in Table 3.

The results are shown in FIGS. 10 and 11. FIGS. 10 and 11 show bands of the reaction mixtures where A101 and A143 were used as the nucleic acid compounds, respectively. In this graph, the concentration (nM) of the nucleic acid compound is indicated on the abscissa, and density in autoradiography determined by a densitometer (i.e. the amount of expressed VEGF) on the ordinate, with "■" in the presence of RNase H and "□" in the absence of RNase H.

As can be seen from these graphs, the inhibitory effect of the oligodeoxyribonucleotide (antisense nucleic acid compound) on the expression of VEGF was reinforced by the coexistent RNase H. That is, the expression of VEGF could be inhibited by the oligodeoxyribonucleotide in lower concentration.

From these results, RNase H was adopted for screening of the desired antisense nucleic acid compound.

(iii) Examination of concentration of nucleic acid compound

The optimum nucleic acid concentration was examined for screening of the desired antisense nucleic acid compound. The experimental method is as follows:

The amount of VEGF expressed in the presence or absence of RNase H with the nucleic acid compound in a concentration ranging from 0 to 2000 nM was determined as described above in (4) (ii). For this experiment, RNase H (11.4 U) and the nucleic acid compound (final concentration: 16 to 2000 nM) were added additionally to the reaction mixture shown in Table 3.

The result is shown in FIG. 12. In this graph, "-●-" indicates the amount of VEGF expressed with A101 as the nucleic acid compound in the presence of 11.4 U RNase H (indicated as "RNase H+"); "-○-", with A101 in the absence of RNase H (indicated as "RNase H-"); "-▲-", with A143 in the presence of 11.4 U RNase H; "-△-", with A143 in absence of RNase H; and "-■-", with RA101 in the presence of 11.4 U RNase H.

Because the inhibitory effect of 0.08 µM or less nucleic acid compound on the expression of VEGF was not significant, 0.4 µM nucleic acid compound was adopted for screening, because with this concentration, the inhibitory effect on the expression of VEGF clearly appeared depending on the type of oligodeoxyribonucleotide.

From the results in (i) to (iii) above, screening was carried out.

The composition of the assay mixture per tube is shown in Table 6.

Table 6

sample	amount (μl)
TNT™ rabbit reticulocyte lysate	12.5
TNT™ reaction buffer	1.0
TNT™ SP6 RNA polymerase	0.5
Amino acid mixture (1 mM; not containing 1 mM methionine)	0.5
Ribonuclease Inhibitor (40 U/μl)	0.5
RNase H (60 U/μl)	0.19
^{35}S-methionine	1.0
Plasmid pSU02 (0.5 μg/μl)	1.0
Nucleic acid compound (5 μM)	2.0
Sterilized water	5.81
Total	25.0

The composition was prepared as described above. The sample was incubated at 37°C for 1 hour. Then, the amount of expressed VEGF was examined by SDS-PAGE and autoradiography as described above in (4) (ii). After autoradiography, the density was measured with a densitometer (Bio-Profil 1-D, M&S Instruments Trading, Inc.) and then compared with a standard curve to evaluate the expression of VEGF as follows:

The produced VEGF was separated by SDS-PAGE and then subjected to autoradiography, and the density was determined. The produced VEGF was estimated by comparing this density with that of a standard curve prepared using the same composition as above but not containing the antisense nucleic acid compound. The result was indicated as a relative value to the amount (as 100 %) of VEGF expressed in the absence of the antisense nucleic acid compound.

To confirm reproducibility, 2 samples were prepared for each compound in the above composition, and the amount of VEGF produced in each sample was determined where 2 lanes were used for each sample.

As a result, the relative error of expression (%) was usually about 10 % or less and maximally about 30 %.

Table 1 shows the effect of each oligodeoxyribonucleotide consisting of 20 nucleotides and Table 2 shows the effect of each oligodeoxyribonucleotide consisting of other than 20 nucleotides in screening for a desired antisense nucleic acid compound. In Tables 1 and 2, a less value in item "expression (%)" means a higher inhibitory effect of the test compound on the expression of the VEGF gene.

Tables 1 and 2 evidently indicate the presence of oligodeoxyribonucleotides inhibiting the expression of VEGF. Among these, there are (1) oligodeoxyribonucleotides having an extremely strong inhibitory effect on the expression of VEGF (inhibiting the amount of expressed VEGF to a level of 10 % or less) and (2) oligodeoxyribonucleotides having a strong inhibitory effect on the expression of VEGF (inhibiting the amount of expressed VEGF to a level of 30 % or less).

As can be seen from Table 1, all 24 oligodeoxyribonucleotides from A383 to A521, 6-nucleotides apart from each other, have an extremely strong inhibitory effect (10 % or less expression). That is, antisense nucleic acid compounds towards some regions within the 383-position or thereabout to the 521-position or thereabout in SEQ ID NO:1 have an extremely strong inhibitory effect (10 % or less expression) on the production of VEGF (said region is hereinafter referred to as "core region".).

The effect of antisense nucleic acid compounds towards partial nucleotide sequences in the core region was examined using different chain lengths.

The nucleotide sequences shown in A422N to A426F, A473N to A473F, and A497N to A505F in Table 2 were examined for their inhibitory effect on the expression of the VEGF gene.

As a result, a chain length of not less than 10 nucleotides gave an extremely strong inhibitory effect (10 % or less expression); the chain length of 6 nucleotides gave no or little inhibitory effect; the chain length of 8 nucleotides gave no effect (1 case), little effect (1 case), or a strong effect (1 case). This different effect of the chain length of 8 nucleotides may be attributable to the difference in the nucleotide sequence or nucleotide composition.

From the results, it is expected that an antisense nucleic acid compound complementary to 10 nucleotides in the core region generally has a strong inhibitory effect on the expression of the target protein (VEGF) and also that an antisense nucleic acid compound complementary to about 8 nucleotides within the core region possibly has a strong inhibitory effect on the expression of the target protein.

From these results and this expectation, it can be estimated that the core regions within the 77- to 570-positions in SEQ NO: 1 are the nucleotide sequences of from the 95- to 108-positions (SEQ ID NO:2), 149- to 174-positions (SEQ ID NO:3), 185- to 210-positions (SEQ ID NO:4), 219- to 244-positions (SEQ ID NO:5), 254- to 276-positions (SEQ ID NO:6), 287- to 328-positions (SEQ ID NO:7), 357- to 372-positions (SEQ ID NO:8), and 389- to 534-positions (SEQ ID NO:9). The core regions are preferably those of SEQ ID NOS:2, 4 and 5, more preferably SEQ ID NOS:6, 7 and 9. An antisense nucleic acid compound complementary to 8 nucleotides, preferably contiguous 8 or more nucleotides, within these core regions shows a significant inhibitory effect on the expression of VEGF, as can be seen from Tables 1 and 2. As the antisense nucleic acid compound complementary to 8 nucleotides in the core regions, mention may be made of antisense nucleic acid compounds A085R, A087P, A089N, A101N, A167N, A179N, A203N, A213N, A237N, A248N, A321N, A365N, and A383N. As the antisense nucleic acid compound complementary to 9 or more nucleotides in the core regions, mention may be made of A089, A095, A095N, A143, A146N, A149, A153N, A155, A155N, A156N, A161, A179, A185, A189N, A191, A191N, A193N, A197, A217N, A227, A251, A251N, A257, A261N, A263, A263N, A265N, A281, A287, A293N, A296N, A299, A299N, A303N, A305, A311, A313N, A317N, A347, A353, A356N, A359, A361N, A397N, A513N, A521N, and 24 antisense nucleic acid compounds of A393 to A521 in Table 1.

Then, the antisense nucleic acid compounds being complementary to nucleotide sequences in the core regions but containing a mismatched nucleotide were examined. As opposed to the known correct base pairs (GC, AT and AU), mismatched base pairs are an AA base pair, AG base pair, AC base pair, GG base pair, GT base pair, GU base pair, CC base pair, CT base pair, CU base pair, TT base pair, TU base pair, and UU base pair. Antisense nucleic acid compounds containing a mismatched nucleotide towards T at the 117-position in SEQ ID NO:9 were synthesized and examined for their inhibitory effect on the expression of VEGF. Table 7 shows antisense compounds each containing one mismatched nucleotide (sample#), their nucleotide sequences and their inhibitory effect on the expression of VEGF (%) as well as on the expression of luciferase as the control. The expression of VEGF (%) was determined by use of the composition shown in Table 6 and expressed as a relative value to the value (100 %) of the composition in the absence of the nucleic acid compound. The expression of luciferase was determined using plasmid pPoly(A)-Luc(SP6) as the control containing the luciferase gene in place of the VEGF structural gene, and is expressed as a relative value to the value (100 %) in the absence of the nucleic acid compound. In item "sample #" in Table 7, the sample given "M" contains a mismatched nucleotide, and the sample given "A" before its number is a antisense chain i.e. a chain complementary to a partial sense chain in the VEGF gene. The number given after "A" indicates the beginning position of its corresponding nucleotide sequence in SEQ ID NO:1. The alphabet given after this number indicates degree of polymerization as follows: L means 12-nucleotides compound; I, 9-nucleotides; K, 11-nucleotides; M, 13-nucleotides; and N, 14-nucleotides. The first number in the parentheses is the number of contiguous complementary nucleotides in the 3'-side, and the latter number is the number of complementary nucleotides in the 5'-side. The alphabet "G" between the numbers in the parentheses is a mismatched nucleotide in the antisense nucleic acid compound. Mismatched nucleotides are underlined in the nucleotide sequences in Table 7. In item "expression of VEGF (%)", the value in the parentheses indicates expression of VEGF (%) in the presence of 50 μM nucleic acid compound (in the case of *1) and 250 μM (in the case of *2) in place of 5 μM in the composition shown in Table 6, and in these cases, the final concentrations of the nucleic acid compound in each reaction solution correspond to 4 μM, and 20 μM, respectively.

Table 7

sample#	nucleotide sequence 5' ← → 3'	expression of VEGF (%)	expression of luciferase (%)
MA489L(7G4)	CTCTGTCTTTCT	4	–
MA489L(7C4)	CTCTCTCTTTCT	53	–
MA501L(4G7)	TTGCTCTGTCTT	0	88
MA501L(4C7)	TTGCTCTCTCTT	0	72
~~MA499L(6G5)~~	~~GCTCTGTCTTTC~~	~~6~~	~~88~~
MA499L(6C5)	GCTCTCTCTTTC	1	50
MA500L(5G6)	TGCTCTGTCTTT	7	76
MA500L(5C6)	TGCTCTCTCTTT	16	62
MA498M(7G5)	GCTCTGTCTTTCT	1	60
MA498M(7C5)	GCTCTCTCTTTCT	1	45
MA500M(5G7)	TTGCTCTGTCTTT	0	78
MA500M(5C7)	TTGCTCTCTCTTT	0	75
MA499M(6G6)	TGCTCTGTCTTTC	0	71
MA499M(6C6)	TGCTCTCTCTTTC	4	53
MA498N(7G6)	TGCTCTGTCTTTCT	1	45
MA498N(7C6)	TGCTCTCTCTTTCT	4	41
MA499N(6G7)	TTGCTCTGTCTTTC	0	71
MA499N(6C7)	TTGCTCTCTCTTTC	0	19
MA502K(5C5)	GCTCTCTCTTT	(0) *1	–
MA501I(4C4)	CTCTCTCTT	(0) *2	–

Based on the results of Table 7, for antisense nucleic acid compounds complementary to core regions with one mismatched nucleotide, those antisense compounds having contiguous at least 5 complementary nucleotides as a shorter sequence interrupted by said mismatched nucleotide and having at the same time at least 11 complementary nucleotides in total (at least 12 nucleotides including one mismatched nucleotide) show the same or almost the same

inhibitory effect as antisense compounds complementary to contiguous at least 8 to 10 nucleotides. Such antisense nucleic acid compounds containing one mismatched nucleotide include MA499L(6G5), MA499L(6C5), MA500L(5G6), MA500L(5C6), MA498M(7G5), MA498M(7C5), MA500M(5G7), MA500M(5C7), MA499M(6G6), MA499M(6C6), MA498N(7G6), MA498N(7C6), MA499N(6G7), and MA499N(6C7). The expression of VEGF can be inhibited by an antisense nucleic acid compound of 11 nucleotides containing one mismatched nucleotide (i.e. with 10 complementary nucleotides) if used at a final concentration of 4 μM or by an antisense nucleic acid compound of 9 nucleotides containing one mismatched nucleotide (i.e. with 8 complementary nucleotides) if used at a final concentration of 20 μM. For example, MA502K(5C5) corresponds to the former case and MA501I(4C4) to the latter case.

As shown above, the expression of VEGF could be inhibited even in the presence of one mismatched nucleotide every 12 nucleotides.

[Test Example]

The inhibitory effect of the phosphorothioate-type oligodeoxyribonucleotide expected to have the antisense nucleic acid effect on the expression of VEGF was examined by screening under the same conditions as above except that 0.8 μM or 1.875 μM nucleic acid compound was used in place of the concentration indicated in Table 6). The results are shown in Table 8.

Table 8

sample#	expression of VEGF (%)	
	64 nM	150 nM
A085R-S	98	57
A087P-S	94	58
A213N-S	71	65
A217N-S	--	33
A227-S	45	--
A237N-S	--	8
A248N-S	--	18
A261N-S	66	50
A287-S	59	--
A299N-S	--	10
A311-S	56	--
A313N-S	--	41
A317N-S	--	39
A321N-S	55	34
A407-S	64	--
A419-S	39	--
A422N-S	--	7
A461-S	28	--
RA143-S	--	78
R3126-S	98	76

In Table 8, "64 nM" is the final concentration of the nucleic acid compound when 0.8 μM nucleic acid compound was used in the composition of Table 6 and "150 nM" means the final concentration when 1.875 μM was used. In Table 8, "S" after the hyphen "-" indicates that the sample is a phosphorothioate type. As is evident from Table 8, the inhibitory effect of the phosphorothioate-type antisense nucleic acid compound on the expression of VEGF was significantly lower than in the presence of the control nucleic acid compound (RA143-S and R3126-S). Because the expression per-

centages of VEGF in the presence of 150 nM RA143-S and R3126-S as the control nucleic acid compounds were 78 % and 76 %, respectively as shown in Table 8, the inhibitory effect of nucleic acid compounds such as A085R-S (57 % expression) and A237N-S (8 % expression) is considered effective. Hence, the phosphorothioate-type oligodeoxyribonucleotide also exhibits the antisense nucleic acid effect. R3126-S in Table 8 has a phosphorothioate-type oligodeoxyribonucleotide as shown in SEQ ID NO:10, which is a random sequence towards the VEGF gene.

The phosphorothioate-type oligodeoxyribonucleotides expected to have the antisense nucleic acid effect were used as the antisense nucleic acid compound to examine their inhibitory effect on the expression of VEGF in cultured cell. This experiment was carried out in a cell culture system using human lung cancer-derived A549 cells under sterized conditions, on the basis of the method described in the literature (M.-Y. Chiang et al., The Journal of Biological Chemistry, Vol. 266, No. 27, pp. 18162 to 18172 (1991)), as follows:

A549 cells were placed in a 48- or 96-well plate and incubated at 37°C under a 5 % CO_2 atmosphere in Dulbecco's modified Eagle medium (DMEM) containing 10 % fetal bovine serum until it became confluent. After removal of the medium, the plate was washed with Opti-MEM medium, and Opti-MEM containing 20 μg/ml lipofectin reagent (available from GIBCO BRL) was put in an amount of 380 μl (when a 48-well plate was used) to each well. 20 μM phosphorothioate-type oligodeoxyribonucleoti de in physiological saline was put in an amount of 20 μl (when a 48-well plate was used) to each well, and the plate was incubated at 37 °C for 4 hours under a 5 % CO_2 atmosphere. The solution was removed, and the plate was washed with DMEM medium containing 10 % fetal bovine serum. Then, DMEM medium containing 10 % fetal bovine serum was put in an amount of 380 μl (when a 48-well plate was used) to each well, and then 20 μM phosphorothioate-type oligodeoxyribonucleotide in physiological saline was put in an amount of 20 μl (when a 48-wells plate was used) to each well. The plate was incubated at 37 °C for 20 hours under a 5 % CO_2 atmosphere. Thereafter, the VEGF in the medium was determined as described above in (4) Confirmation of expression of VEGF, (i) Enzyme immunoassays. The expression of VEGF in the presence of the phosphorothioate-type oligodeoxyribonucleotide was determined by making a comparison with the expression (assumed to be 100 %) of VEGF in the absence of said compound. The results are shown in Table 9.

Table 9

sample#	expression of VEGF (%)
A085R-S	63±5
A087P-S	65±1
A227-S	70±4
A287-S	60±0
A311-S	66±6
A419-S	54±9
S085R-S	92±1
RA143-S	85±1

As shown in Table 9, the amount of VEGF expressed in the presence of the phosphorothioate-type oligodeoxyribonucleotide as the antisense nucleic acid is lower than in the presence of the phosphorothioate-type oligodeoxyribonucleotide of the random sequence (RA143-s) or the sense sequence (S085R-S). That is, because as shown in Table 2, the expression percentages of VEGF in the presence of S085R-S and RA143-S as the control nucleic acids were 92 % and 85 %, respectively, the inhibitory effect of samples such as A085R-S (63 % expression) and A419-S (54 % expression) etc. is considered to be effective.

From the foregoing, it was found that the phosphorothioate-type oligodeoxyribonucleotides having the nucleotide sequences selected in the screening in the cell-free system can be used to inhibit the expression of VEGF in the cultured cells, as well.

The phosphorothioate-type oligodeoxyribonucleotides expected to have the antisense nucleic acid effect were used as the antisense nucleic acid to examine their inhibitory effect on the expression of VEGF in animals. As the indicator of the inhibitory effect, the growth of human fibroblast-derived HT1080 cells transplanted intracutaneously in nude mice was used as described below. 1×10^6 HT1080 cells derived from human fibroblast, obtained by tissue culture in Dulbecco's modified Eagle medium (DMEM), were transplanted intracutaneously in each 4-week-old male nude mouse (BALB/C nu/nu) (Day 0). 100 μl each of 100 μM phosphorothioate-type nucleic acid compound A419-S in physiological saline, 100 μM phosphorothioate-type nucleic acid compound RA419-S in physiological saline, and physiological saline

only were given respectively to mice once per day. A419-S is the same compound designated A419-S in Table 8, and its nucleotide sequence is shown in A419 in Table 1. RA419-S is identical in nucleotide composition with A419-S in Table 1, but not complementary to the nucleotide sequence of the VEGF gene, and its nucleotide sequence is shown in SEQ ID NO:11.

After intracutaneous transplantation of HT1080 cells, each nude mouse was examined every about 3 days for body weight and tumor size and simultaneously a picture was taken. The group given the antisense nucleic acid compound (A419-S) indicated a smaller tumor size than the group given RA419-S or physiological saline. As an example, the results on Day 19 are shown in Table 10.

Table 10

	tumor size (19 days after transplantation)
A419-S	740±150 mm^3 (n = 3)
RA419-S	1470±870 mm^3 (n = 3)
control (physiological saline)	1260±320 mm^3 (n = 2)

From the foregoing, it was found that the phosphorothioate-type oligodeoxyribonucleotide selected by screening in the cell-free and cultured-cell systems can be used to inhibit tumor growth in experimental animals. This inhibition of tumor growth can be considered to result from the inhibitory effect of the antisense nucleic acid on the expression of VEGF.

From a large number of these examples, it could be estimated that the antisense nucleic compound having a nucleotide sequence complementary to 8 or more nucleotides within the core region exerts a strong inhibitory effect on the expression of VEGF. In view of the role of VEGF as a tumor angiogenic factor in vivo (K. J. Kim et al., Nature, Vol. 362, April 29 issue, pp. 841 - 844 (1993); and S. Kondo et al., Biochemical and Biophysical Research Communications, Vol. 194, No. 3, pp. 1234 - 1241 (1993)), the antisense nucleic acid compound having a nucleotide sequence complementary to 8 or more nucleotides in the core region is useful as a therapeutic agent such as anticancer drug to inhibit the growth of solid tumor cells or as a diagnostic agent for cancers.

Industrial Applicability

According to the present invention, there is provided an antisense nucleic acid compound inhibiting the expression of the gene coding for VEGF.

The antisense nucleic acid compound of the present invention can inhibit the growth of solid tumors by inhibiting the expression of VEGF i.e. a factor facilitating the arrival of blood vessels at the solid tumors. Therefore, it can be used as an anticancer drug to inhibit the growth of solid tumor cells. Because VEGF is involved in rheumatoid arthritis and diabetes as well, the antisense nucleic acid compound of the present invention can also be used as an therapeutic agent for such diseases.

Further, the antisense nucleic acid compound of the present invention can be used as a diagnostic agent for detection of solid tumor cells as well as for diagnosis of rheumatoid arthritis and diabetes.

SEQUENCE LISTING

SEQ ID NO: 1:

LENGTH: 774 bases

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: cDNA to mRNA

SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TTATGTATCA TACACATACG ATTTAGGTGA CACTATAGAA TACAAGCTTA TGCATGCGGC 60

CGCATCTAGA GGGCCCGGCC CCGGTCGGGC CTCCGAAACC ATGAACTTTC TGCTGTCTTG 120

GGTGCATTGG AGCCTTGCCT TGCTGCTCTA CCTCCACCAT GCCAAGTGGT CCCAGGCTGC 180

ACCCATGGCA GAAGGAGGAG GGCAGAATCA TCACGAAGTG GTGAAGTTCA TGGATGTCTA 240

TCAGCGCAGC TACTGCCATC CAATCGAGAC CCTGGTGGAC ATCTTCCAGG AGTACCCTGA 300

TGAGATCGAG TACATCTTCA AGCCATCCTG TGTGCCCCTG ATGCGATGCG GGGGCTGCTG 360

CAATGACGAG GGCCTGGAGT GTGTGCCCAC TGAGGAGTCC AACATCACCA TGCAGATTAT 420

GCGGATCAAA CCTCACCAAG GCCAGCACAT AGGAGAGATG AGCTTCCTAC AGCACAACAA 480

ATGTGAATGC AGACCAAAGA AAGATAGAGC AAGACAAGAA AAATGTGACA AGCCGAGGCG 540

GTGAGCCGGG CAGGAGGAAG GAGCCTCCCT CAGGGTTTCG GGAACCAGAT CCACTAGTTC 600

TAGATGCATG CTCGAGCGGC CGCCAGTGTG ATGGATATCT GCAGAATTCC AGCACACTGG 660

CCGTTACTAG TGGATCCGAG CTCCCAAAAA AAAAAAAAAA AAAAAAAAAA AAAAACCGAA 720

TTAATTCGTA ATCATGGTCA TAGCTGTTTC CTGTGTGAAA TTGTTATCCG CTCA 774

SEQ ID NO: 2:

LENGTH: 14 bases

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: cDNA to mRNA

SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GAAACCATGA ACTT

SEQ ID NO: 3:

LENGTH: 26 bases

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: cDNA to mRNA

SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TACCTCCACC ATGCCAAGTG GTCCCA

SEQ ID NO: 4:

LENGTH: 26 bases

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: cDNA to mRNA

SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ATGGCAGAAG GAGGAGGGCA GAATCA

SEQ ID NO: 5:

LENGTH: 26 bases

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: cDNA to mRNA

SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TGGTGAAGTT CATGGATGTC TATCAG

SEQ ID NO: 6:

LENGTH: 23 bases

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: cDNA to mRNA

SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TGCCATCCAA TCGAGACCCT GGT

SEQ ID NO: 7:

LENGTH: 38 bases

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: cDNA to mRNA

SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CAGGAGTACC CTGATGAGAT CGAGTACATC TTCAAGCCAT CC

SEQ ID NO: 8:

LENGTH: 16 bases

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: cDNA to mRNA

SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GCTGCAATGA CGAGGG

SEQ ID NO: 9:

LENGTH: 146 bases

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: cDNA to mRNA

SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ACTGAGGAGT CCAACATCAC CATGCAGATT ATGCGGATCA AACCTCACCA AGGCCAGCAC 60

ATAGGAGAGA TGAGCTTCCT ACAGCACAAC AAATGTGAAT GCAGACCAAA GAAAGATAGA 120

GCAAGACAAG AAAAATGTGA CAAGCC 146

SEQ ID NO: 10:

LENGTH: 20 bases

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AAAAAAACAA AAACAACAAA

SEQ ID NO: 11:

LENGTH: 20 bases

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CTAGACTGTG TGTTCTGGAG

Claims

1. An antisense nucleic acid compound which has a nucleotide sequence complementary to at least 8 contiguous nucleotides in the nucleotide sequence of a gene coding for a vascular endothelial growth factor and which inhibits the expression of the vascular endothelial growth factor to 30 % or less.

2. An antisense nucleic acid compound according to claim 1 wherein the gene coding for the vascular endothelial growth factor is SEQ ID NO:1.

3. An antisense nucleic acid compound according to claim 1 or 2 wherein the expression of the vascular endothelial growth factor is 10 % or less in the presence of the compound as compared to the expression in the absence of the compound.

4. An antisense nucleic acid compound which has a nucleotide sequence complementary to at least 8 contiguous nucleotides in any of the nucleotide sequences of SEQ ID NOS: 3 to 9 and which inhibits the expression of the vascular endothelial growth factor.

5. An antisense nucleic acid compound which has a nucleotide sequence complementary to at least 10 contiguous nucleotides in any of the nucleotide sequences of SEQ ID NOS: 2 to 9 and which inhibits the expression of the vascular endothelial growth factor.

6. An antisense nucleic acid compound according to claim 4 or 5 wherein the nucleotide sequence of the antisense nucleic acid compound has a complementary nucleotide sequence of at least 8 nucleotides and the number of nucleotides in the antisense nucleic acid compound ranges from 14 to 30.

7. An antisense nucleic acid compound according to claim 6 wherein the antisense nucleic acid compound of 14 to 30 nucleotides consists of a complementary nucleotide sequence.

8. An antisense nucleic acid compound which has a nucleotide sequence being partially complementary to, and having one nucleotide complementarily mismatched to, 9 or more contiguous nucleotides in any of the nucleotides of SEQ ID NOS: 2 to 9, and which inhibits the expression of the vascular endothelial growth factor.

9. An antisense nucleic acid compound which has 12 or more nucleotides having 11 or more nucleotides complementary to, and 1 nucleotide complementarily mismatched to, any of the nucleotides of SEQ ID NOS: 2 to 9 and which inhibits the expression of the vascular endothelial growth factor, wherein the shorter contiguous chain interrupted by said mismatched nucleotide consists of 5 or more nucleotides

10. A therapeutic agent comprising as active ingredient an antisense nucleic acid compound according to any one of claims 1 to 9 and a pharmaceutically acceptable carrier.

11. A diagnostic agent comprising as active ingredient an antisense nucleic acid compound according to any one of claims 1 to 9 and a pharmaceutically acceptable carrier.

12. A method of preventing the expression of a vascular endothelial growth factor, wherein a nucleotide sequence complementary to at least 8 contiguous nucleotides in the nucleotide sequence of a gene coding for the vascular endothelial growth factor is used to inhibit the expression of the vascular endothelial growth factor to 30 % or less.

FIG. 1

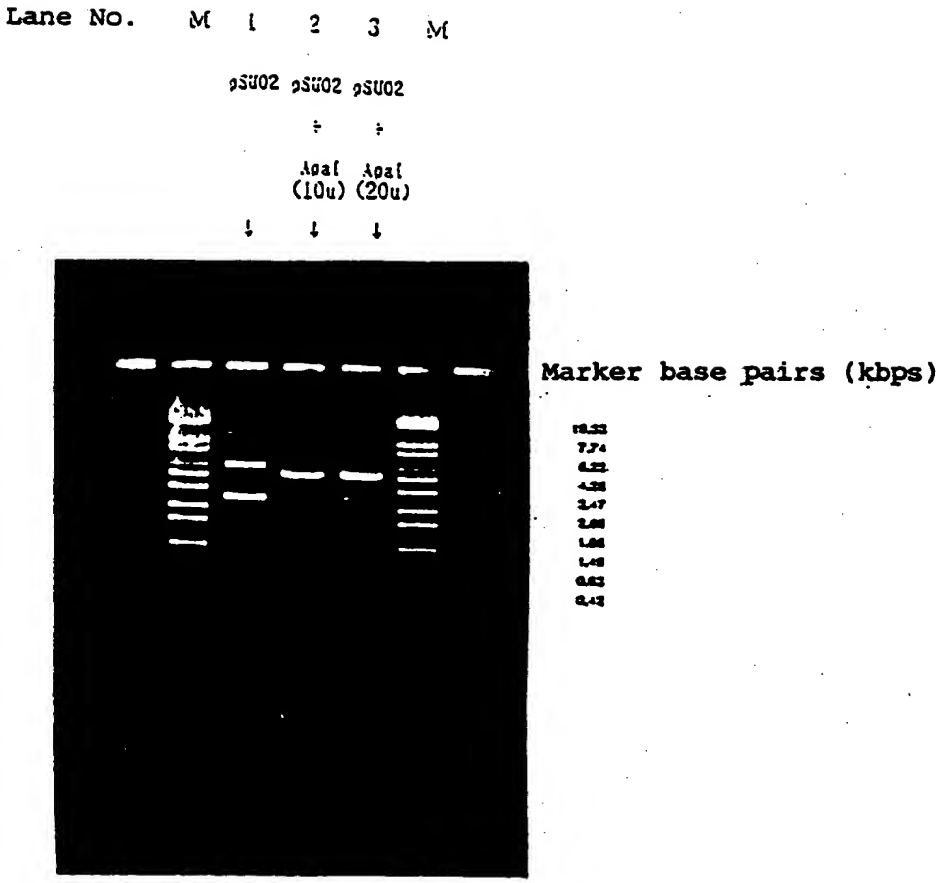


FIG.2

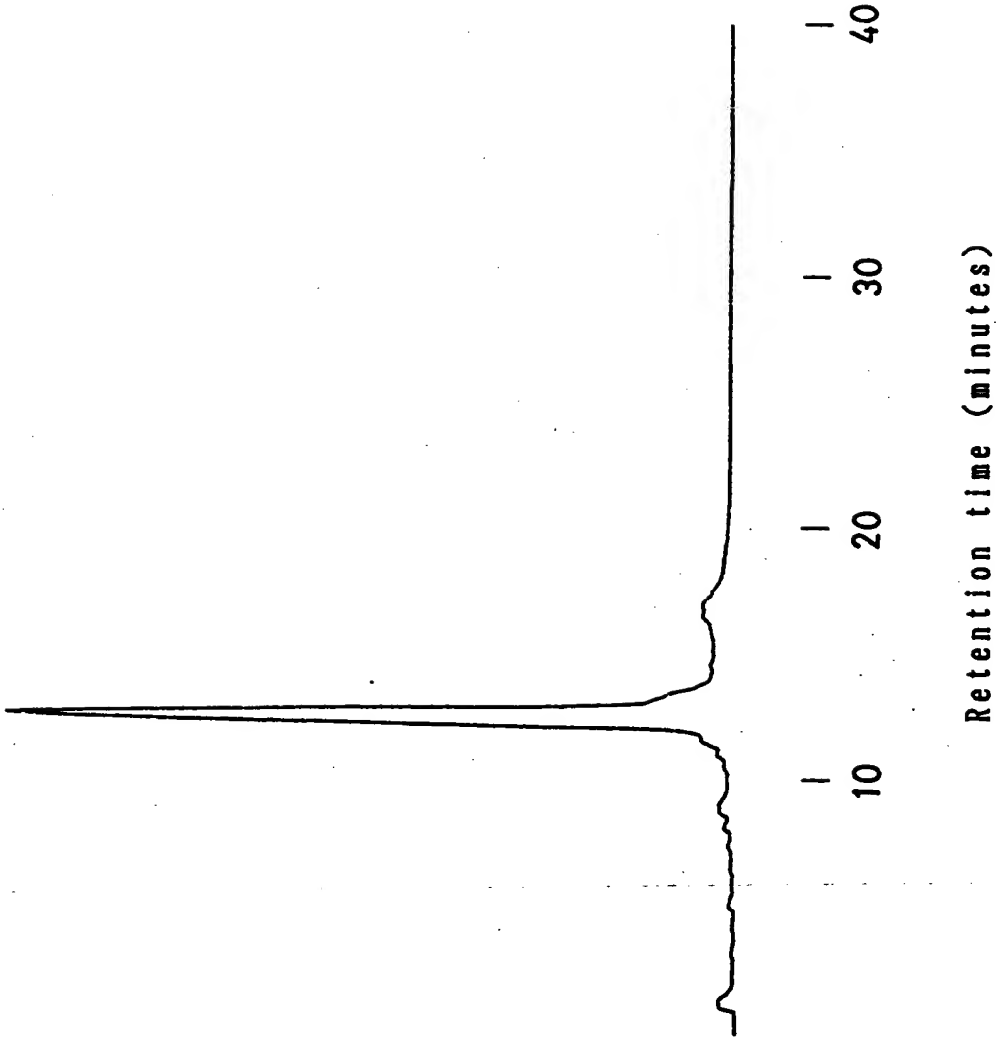


FIG. 3

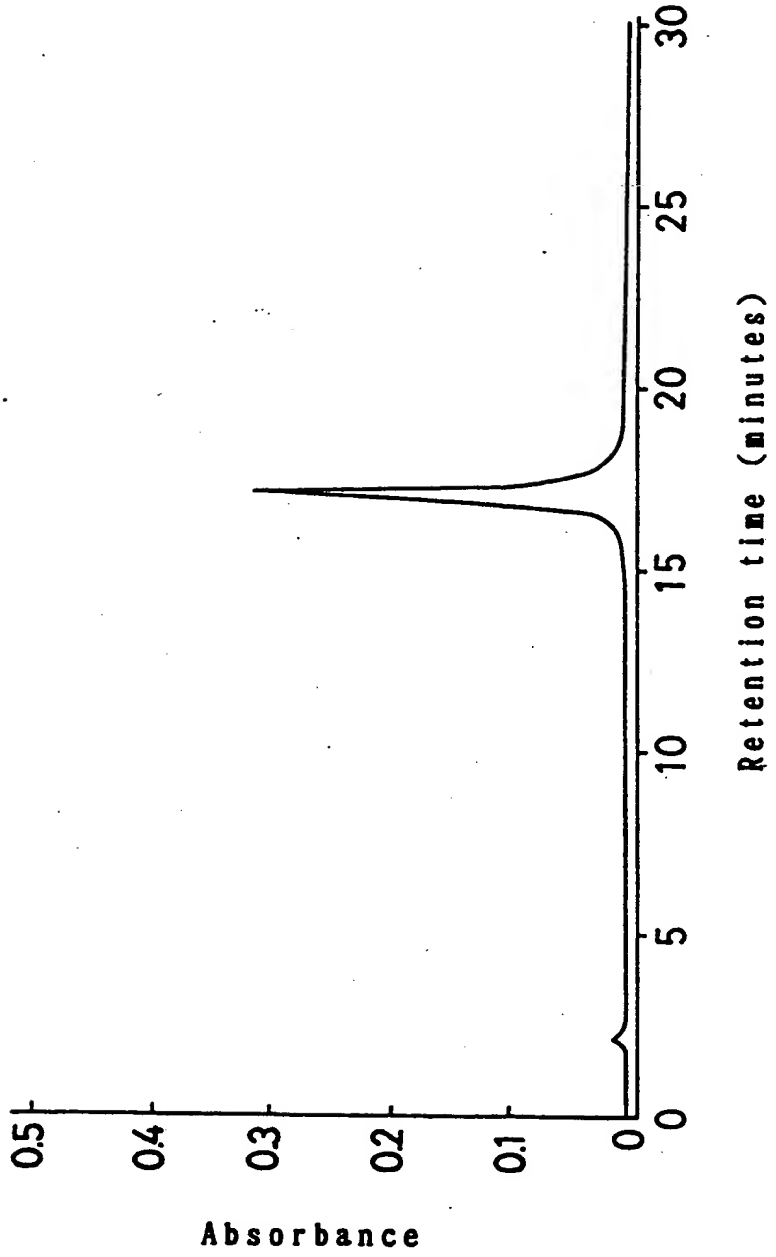


FIG. 4

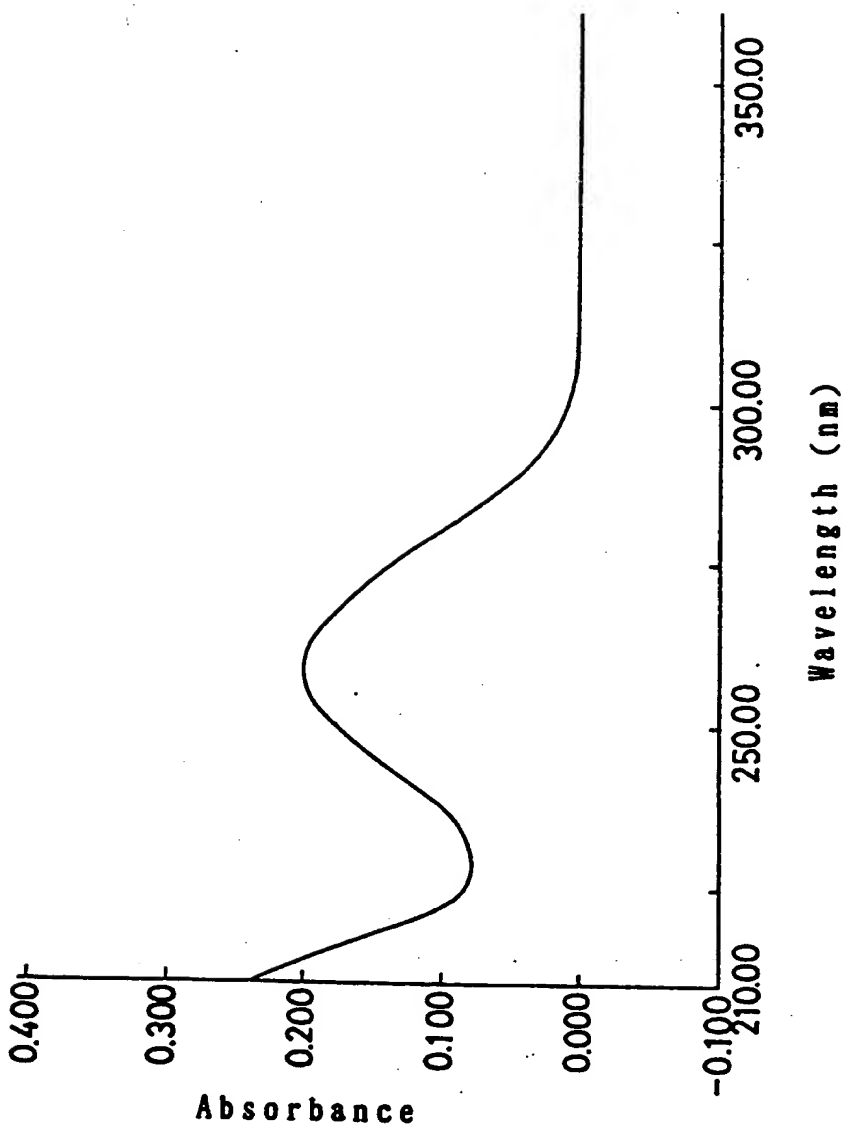

0.400
0.300
0.200
0.100
0.000
-0.100
Absorbance
210.00
250.00
300.00
350.00
Wavelength (nm)

FIG.5

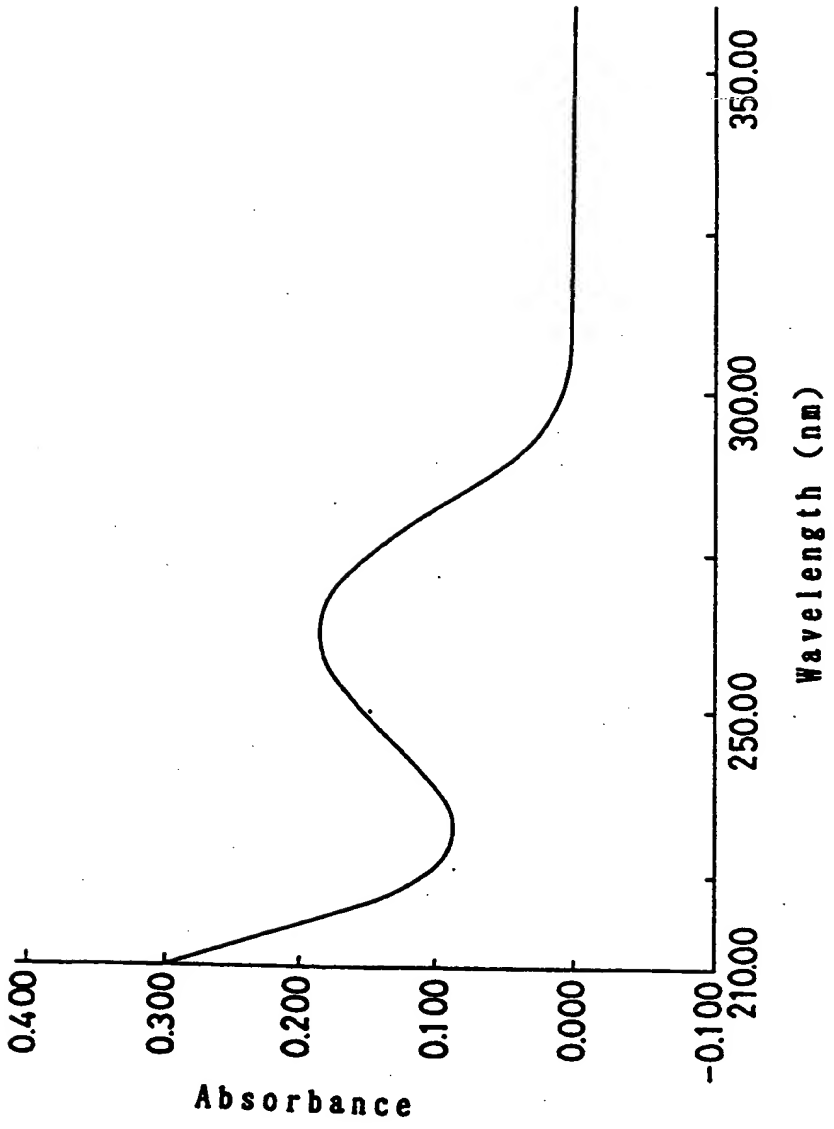


FIG.6

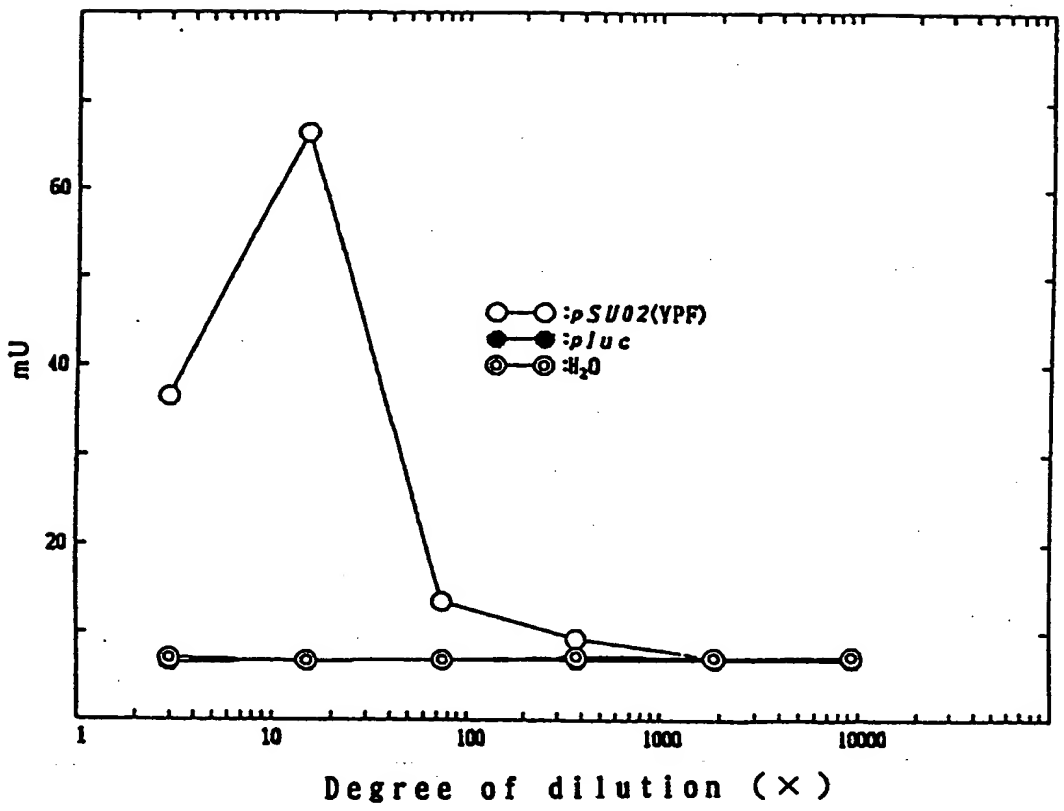

mU
60
40
20
1
10
100
1000
10000
Degree of dilution (×)
:pSV02(YPF)
:pluc
:H2O

FIG.7

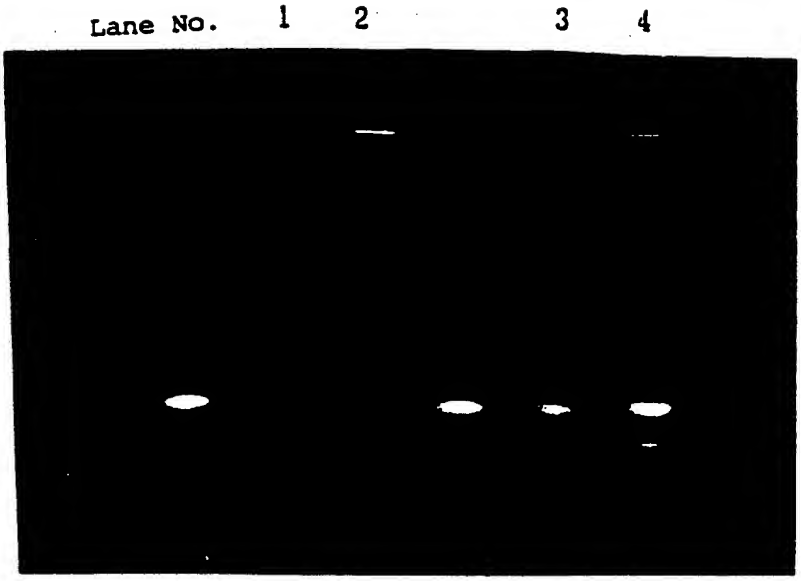


FIG.8

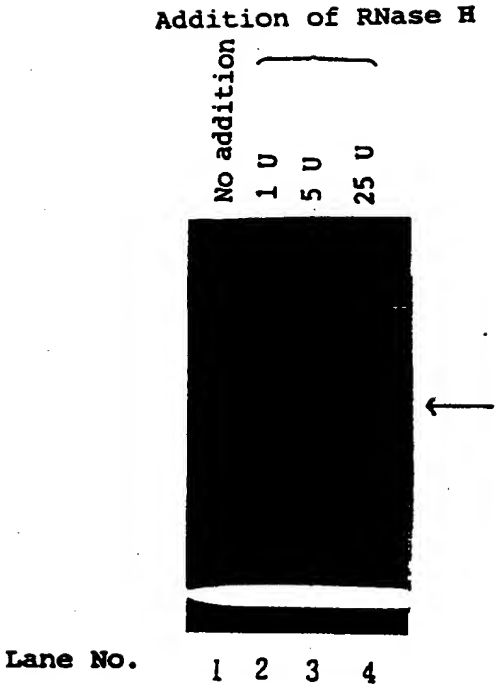


FIG.9

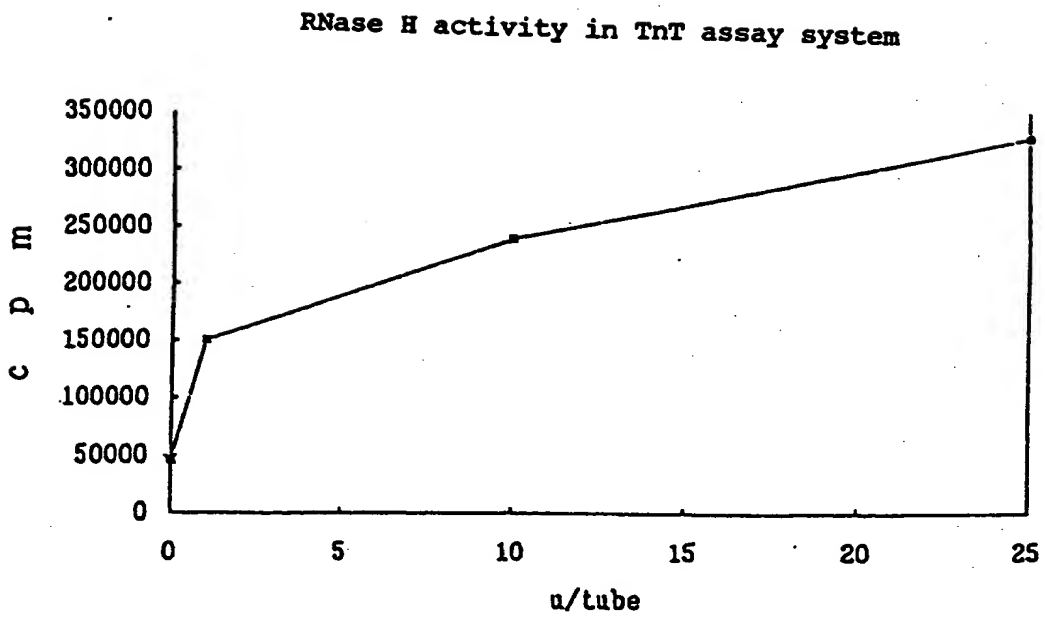


FIG. 10

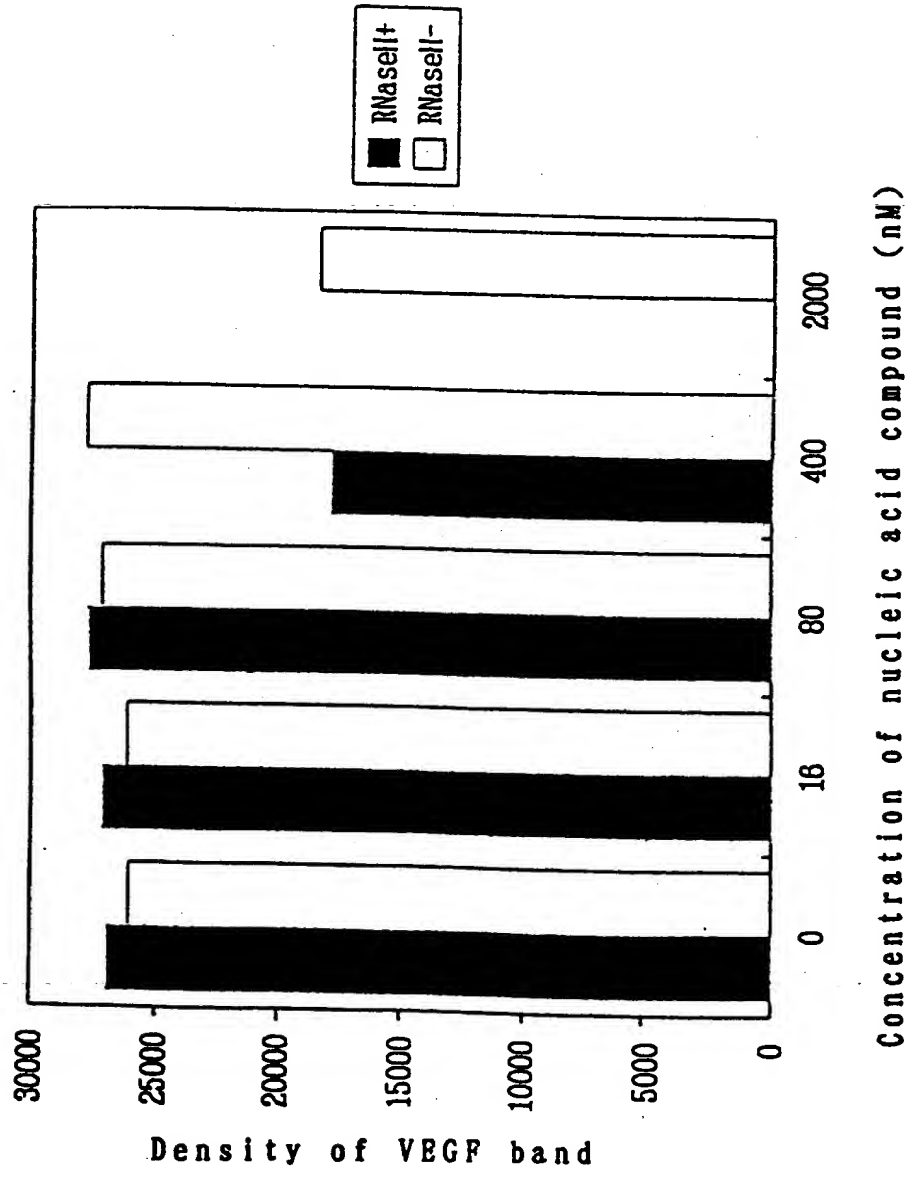

RNaseII+
RNaseII-
30000
25000
20000
15000
10000
5000
0
Density of VEGF band
0
16
80
400
2000
Concentration of nucleic acid compound (nM)

FIG. 11

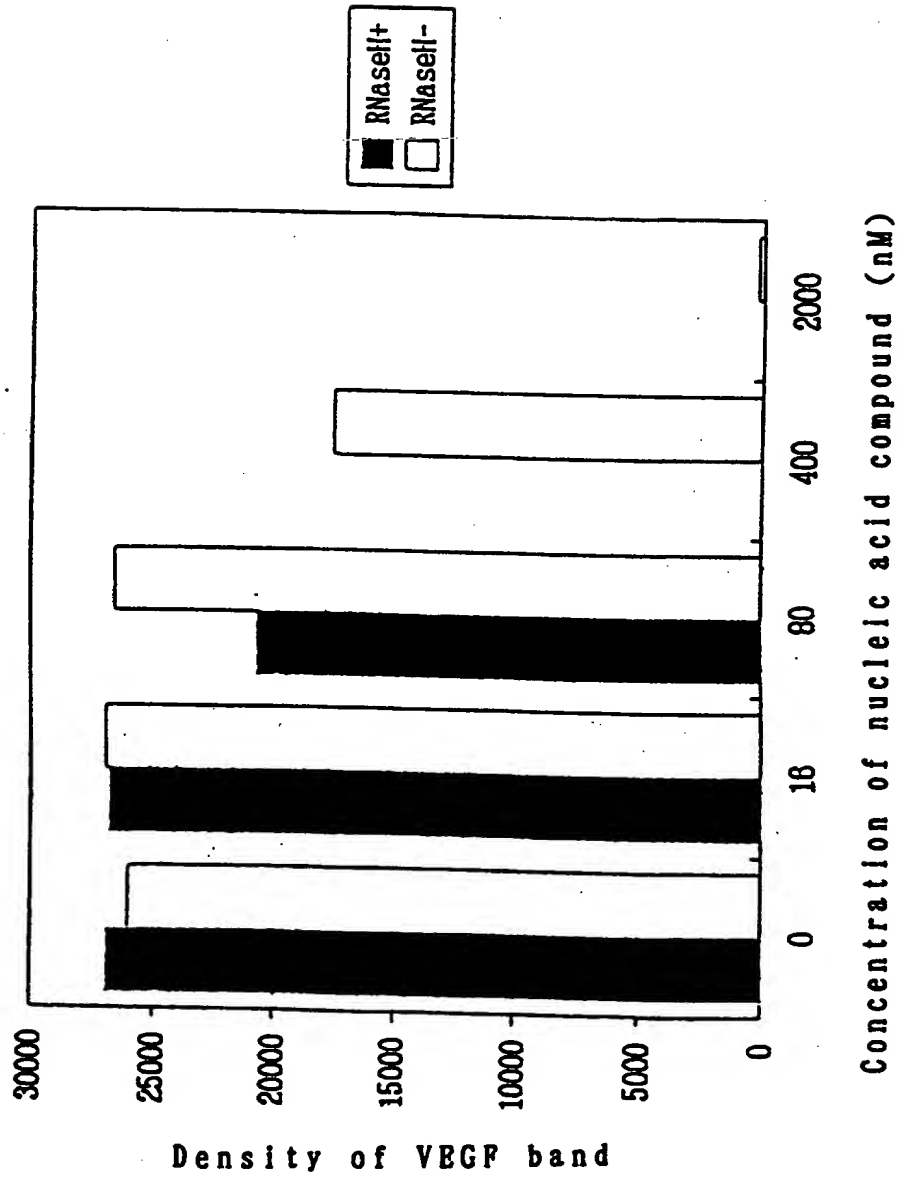
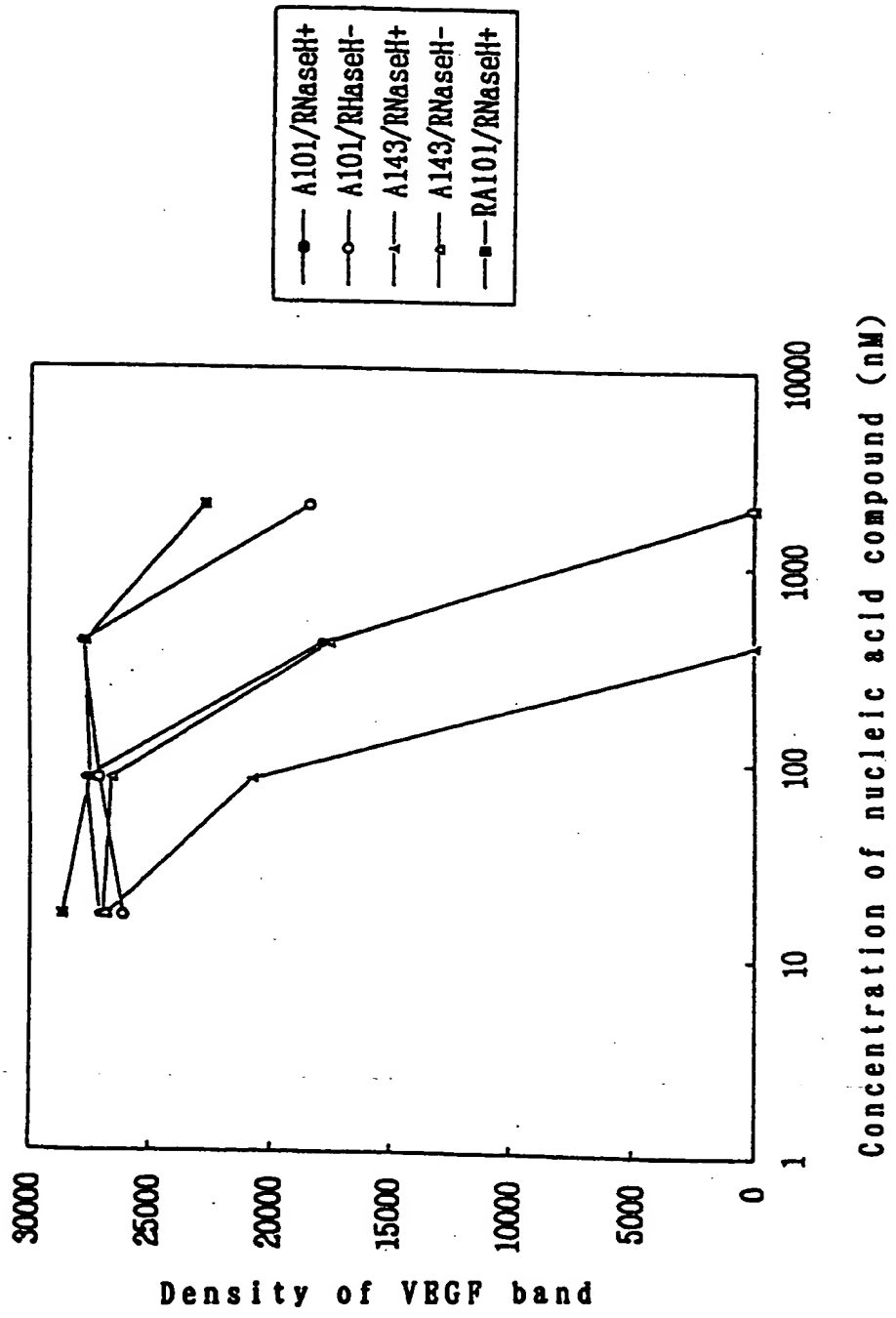


FIG. 12


A101/RNaseH+
A101/RHaseH-
A143/RNaseH+
A143/RNaseH-
RA101/RNaseH+
Concentration of nucleic acid compound (nM)
10000
1000
100
10
1
30000
25000
20000
15000
10000
5000
0
Density of VEGF band

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP95/01121

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl^6 C12N15/11, A61K48/00, G01N33/50
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
Int. Cl^6 C12N15/11, A61K48/00, G01N33/50

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAS ONLINE, WPI, WPI/L, BIOSIS PREVIEWS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO, 95/04142, A2 (HYBRIDON INC.), February 9, 1995 (09. 02. 95) & AU, 9475168, A	1 - 12
A	Kim, K. J. et al. "Inhibition of vascular endothelial growth factor-induced engiogenesis suppresses tumour growth in-vivo", Nature (1993), Vol. 362, No. 6423, p. 841-844	1 - 12
A	Kondo, S. et al. "Significance of vascular endothelial growth factor-vascular permeability factor for solid tumor growth and its inhibition by the antibody" Biochem. Biophys. Res. Commun. (1993), Vol. 194, No. 3 p. 1234-1241	1 - 12
A	Tischer, E. et al. "The human gene for vascular endothelial growth factor", J. Biol. Chem. (1991), Vol. 266, No. 18, p. 11947-11954	1 - 12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
August 15, 1995 (15. 08. 95)	September 5, 1995 (05. 09. 95)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP95/01121

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO, 91/02058, A (California Biotechnology Inc.), February 21, 1991 (21. 02. 91) & AU, 9060798, A & EP, 484401, A & US, 5194596, A & JP, 5-501350, A	1 - 12

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